Universidad Autónoma de Madrid

Facultad de Medicina

Departamento de Obstetricia y Ginecología

TESIS DOCTORAL

PREDICCIÓN MULTIVARIANTE DEL SÍNDROME DE RESPUESTA INFLAMATORIA FETAL A PARTIR DEL USO COMBINADO DE MARCADORES BIOQUÍMICOS

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Director:

José Luis Bartha Rasero

Madrid, 2017.

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Al Dr. Daniel Abehsera, mi residente mayor, amigo y primer mentor en el mundo de la Obstetricia. Esta tesis se la debo a él, no sólo por incluirme en el proyecto hace ya muchos años, si no por ser el primero en creer en mí y motivarme a dar los pasos que me han llevado hasta donde estoyahora.

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RESUMEN DE:

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INTRODUCCIÓN Y OBJETIVOS.

El parto pretérmino es aún un problema sin resolver en medicina perinatal y aunque su etiología es compleja, el complejo infección-inflamación parece tener un peso importante en su origen, especialmente cuando se habla de edades gestacionales muy tempranas.

El origen de esta infección en la mayoría de los casos es debido al ascenso de microorganismos del tracto genital inferior. Existen múltiples gérmenes implicados en el desarrollo de una infección intraamniótica sin embargo Ureaplasma urealyticum es el microorganismo más comúnmente aislado en casos de infección intraamniótica.

La invasión microbiana de la cavidad amniótica produce la liberación de mediadores inflamatorios que condicionan una ambiente de inflamación intrauterina. Este proceso puede desencadenar la aparición de corioamnionitis histológica y afectar al componente fetal de la gestación desencadenando una funisitis y produciendo lesiones especialmente en el sistema nervioso central y sepsis fetal.

De tal manera que la inflamación que ocurre en el contexto de una infección intraamniótica es un fenómeno variable, probablemente con diferentes grados de severidad y que puede llegar a culminar en lesiones neurológicas neonatales y sepsis fetal.

A la hora de evaluar las posibles consecuencias maternas y fetales de una infección intraamniótica es esencial de disponer de herramientas que nos ayuden a diferenciar entre los diferentes estadíos de inflamación intrauterina y que nos permitar dar un pronóstico lo más certero posible que nos ayude a un mejor manejo de la gestación.

Muchos estudios relacionan diferentes citoquinas proinflamatorias con parto pretérmino, corioamnionitis y morbimortalidad materna. Sin embargo hasta la fecha no existe unos marcadores establecidos en la práctica clínica que nos permitan diferenciar entre los diferentes estadíos de inflamación intrauterina.

El objetivo de la presente tesis doctoral es encontrar encontrar un perfil de marcadores inflamatorios en líquido amniótico que identifiquen y diferencien entre los diferentes estados de inflamación materno y fetal en el contexto de una infección intraamniótica.

METODOLOGÍA

Estudio prospectivo en el que se estudian pacientes en riesgo de infección intraamniótica. Se catalogan como pacientes en riesgo aquellas diagnosticadas de Rotura prematura de membranas (RPM) y/o Amenaza de parto pretérmino (APP) entre la semana 24 y 32 de gestación.

Los criterios de exclusión son: diagnóstico de gestación gemelar, la presencia de un trabajo de parto establecido y la presencia de corioamnionitis clínica.

Las pacientes serán informadas de la posibilidad de realizar una amniocentesis diagnóstica con el fin de poner de manifiesto la presencia de una infección intraamniótica. Durante la amniocentesis se extraían 10 cc de líquido amniótico (LA) para el presente estudio: 6 cc para cultivo microbiológico y 2 cc para estudio mediante PCR. Posteriormente se congelaban 2cc de LA para el estudio ulterior de citoquinas y moléculas proinflamatorias.

Posteriormente al parto se llevará a cabo un examen de la placenta. Aquellos casos en los que se objetivara la presencia de lesiones inflamatorias serán clasificadas en: corioamnionitis aguda, como resultado de una lesión inflamatoria materna o bien como corioamnionitis complicada con funisitis necrotizante de acuerdo a la existencia de una respuesta inflamatoria fetal.

Las citoquinas y marcadores inflamatorios analizados son: IL-1b, IL-2, IL-4, IL-6, IL 8, IL-10, IL-12, TNF-alpha, IFN- gamma, y MMP-8 mediante la técnica de Multiplex.

Las muestras de LA posteriormente se procesan para el estudio de patrones de metabólicos específicos.

Posteriormente se analizarán los resultados anatomopatologicos de las placentas, el microbiológico del líquido amniótico, los niveles de interleuquinas inflamatorias y finalmente los resultados clínicos tanto de la gestación como del neonato.

Los resultados obtenidos se analizarán mediante el programa SPSS 15.0 (SPSS, Chicago IL, USA) utilizando correlación lineal, t test y análisis de regresión logística. El nivel de significatividad previamente establecido es del 95% ($p < 0.05$).

INVESTIGACIONES REALIZADAS

Los tres estudios que conforman la presente tesis forman parte de la misma línea de investigación basada en la identificación de biomarcadores en líquido amniótico capaces de predecir y diferenciar entre los diferentes estadíos de inflamación intrauterina.

ESTUDIO 1

Differential amniotic fluid cytokine profile in women with chorioamnionitis with and without funisitis. Revello R, Alcaide MJ, Dudzik D, Abehsera D, Bartha JL. J Matern Fetal Neonatal Med. 2016;29(13):2161-5

ESTUDIO 2

LC-MS-based metabolomics identification of novel biomarkers of chorioamnionitis and its associated perinatal neurological damage. Dudzik D, Revello R, Barbas C, Bartha JL. J Proteome Res. 2015 Mar 6;14(3):1432-44.

ESTUDIO 3

Prediction of Chorioamnionitis in Cases of Intraamniotic Infection by Ureaplasma Urealyticum in Women with Very Preterm Premature Rupture of Membranes or Preterm Labour. Revello R, Alcaide MJ, Abehsera D, Martin-Camean M, Sousa E Faro Gomes M, Alonso-Luque B, Bartha JL. J Matern Fetal Neonatal Med. 2017 May 14:1-13.

RESULTADOS

El objetivo del ESTUDIO 1 era Identificar una combinación de citoquinas inflamatorias capaces de diferenciar entre la presencia de corioamninonitis y corioamninitis más funsitis, en aquellas pacientes con alto riesgo de infección intraamniótica.

El estudio incluyó 40 pacientes con riesgo de infección intraamniótica. 12 (30%) fueron casos de APP con membranas íntegras y 28 (70%) RPM pretérmino.

Se llevó a cabo un análisis estadístico para analizar los niveles de diferentes citoquinas inflamatorias en líquido amniótico (IL-1b, IL-2, IL-4, IL-6, IL 8, IL-10, IL-12, TNF-alpha, IFN-gamma, y MMP-8) de estas pacientes. Se realizó 3 tipos de comparaciones:

- Comparación entre el grupo sin lesión histológica (grupo control) con el grupo de corioamnionitis aislada.

- Comparación entre el grupo control con el grupo de corioamnionitis + funisitis.

- Comparación entre el grupo de corioamnionitis aislada con corioamnionitis+ funisitis.

Sólo IL-6, IL-12, IL-8, and MMP-8 presentación diferencias estadísticamente significativas en la comparación entre grupo control y corioamnionitis aislada. Sin embargo, cuando se realizó la comparación entre grupo control y el grupo de corioamnionitis + funisitis se encontraron diferencias en la mayoría de las citoquinas estudiadas: IL-1b, IL-2, IL-6, IL- 10, IL-12, IL-8, TNF-alpha, y MMP-8.

Se encontraron diferencias estadísticamente significativas en las citoquinas: IL-1b, IL-6, IL-10, IL-12, IL-8, y TNF-alpha cuando se comparó los grupo de corioamnionitis aislada y corioamnionitis + funisitis.

El análisis de regresión logística binaria aportó un modelo de predicción de funisitis con un alto valor predictivo ($R2= 1$, $p < 0.00001$) y que incluía en la ecuación las citoquinas IL-4, IL-8, IL-10, and IL-12.

El objetivo del ESTUDIO 2 era identificar biomarcadores con el estudio metabólico del líquido amniótico en pacientes con alto riesgo de infección intraamniótica, capaces de predecir aquellas pacientes con corioamnionitis y, dentro de ellas, aquellas en las que se desarrollaría lesión neurológica perinatal.

Los resultados del presente estudio mostraron que:

- La acumulación de ciertas ceramidas en líquido amniótico relacionadas con procesos apoptóticos en las membranas amnióticas son marcadores sensibles de

corioamnionitis. El grupo de los esfingolípidos , especialmente esfingomielina y lactosilceramidas fueron los metabolitos que diferenciaban aquellas pacientes con corioamnionitis

- El aumento de algunos ácidos biliares en un ambiente proinflamatorio podría ser importante en la predicción y la detección de aquellos neonatos con alto riesgo de desarrollar hemorragia interventricular.
- Lisofosfatidilcolina, el ácido sulfocólico y especialmente el ácido trioxocolenoico podrían ser considerados como posibles marcadores diagnósticos en la identificación de neonatos con daño neurológico perinatal asociado a corioamnionitis materna.

De tal manera que, estos resultados sugieren que la metabolómica es una herramienta sensible para la identificación de un perfil de metabolitos en líquido amniótico relacionado con la infección intraamniótica y podría a ser crucial a la hora de identificar biomarcadores diagnósticos y terapéuticos de coriomanionitis y lesión neurológica perinatal.

El objetivo del ESTUDIO 3 era determinar la capacidad predictiva de determinadas citoquinas inflamatorias para el diagnóstico de corioamnionitis en casos de infección intraamniótica por Ureaplasma urealyticum. En primer lugar porque Ureaplasma es el microorganismo más comúnmente aislado en casos de infección intraamniótica y en segundo lugar, porque actualmente existe la creencia de que este microorganismo de baja virulencia puede llegar, en algunos casos, a colonizar el especio coriodecidual sin llegar a progresar a niveles más severos de inflamación.

Nuestros resultados fueron acordes a los datos publicados anteriormente. De las 20 pacientes en las que se objetivó infección amniótica por Ureaplasma urealyticum menos de la mitad de los casos desarrollaron corioamnionitis histológica, el 45%, mientras que tan sólo 3 de los casos (15%) llego a desarrollar funisitis.

De todas las citoquinas estudiadas, TNF-alpha/IFN-gamma/IL-1b/IL-2/IL-6/IL-8/IL-12/IL-4/IL-10 y MMP-8, tan sólo IL6, IL 8, IL12 y MMP8 presentaron diferencias significativas entre los grupos con y sin inflamación histológica.

De todas estas moléculas, en casos de infección por Ureasplama, IL 6 fue la que presentó más capacidad predictiva de corioamnionitis. En nuestra muestra, unos valores superiores a 400 pg/ml detectarían un 100% de los casos de corioamnionitis, mientras que unos valores superiores a 900 pg/ml aumentaría la especificidad hasta el 100% con una tasa de detección del 75%.

Como objetivo secundario del estudio buscábamos analizar el efecto de la terapia con Azitromicina en pacientes con RPM pretérmino e infección intraamniótica por Ureaplasma. De los 16 casos tratados con Azitromicina, la mitad desarrolló corioamnionitis histológica aislada y la otra mitad no presentaron lesiones histológicas. Ningún caso tratado con Azitromicina desarrollo funisitis.

CONCLUSIONES

- El proceso de inflamación intraamniótica es un proceso variable con diferentes grados de severidad. Entre las pacientes con riesgo de infección intraamniótica, esto es, rotura prematura de membranas y amenaza de parto pretérmino con factores de mal pronóstico, se desarrolló una inflamación histológica en un 37.5% de las pacientes, sin embargo, tan sólo una pequeña proporción de casos, un 15%, evolucionó a funisitis, la expresión histológica del síndrome de respuesta inflamatoria fetal.
- La causa subyacente de estos procesos de inflamación intrauterina es la infección intraamniótica en la mayoría de los casos, un 80% de los casos en nuestra serie.
- La combinación de IL 12, 10, 4 y 8 en líquido amniótico tiene un alto valor predictivo para la detección de funisitis en pacientes con riesgo de infección intraamniótica.
- En un futuro, puede que sea la combinación de citoquinas inflamatorias, y no la determinación de una IL aislada la que nos lleve a una identificación de los diferentes grados de inflamación histológica en el contexto de una infección intraamniótica.

- La metabólomica es una nueva técnica que aunque prometedora, aún no ha sido ampliamente estudiada en el contexto de la corioamnionitis subclínica y sus consecuencias perinatales. Se necesitan más estudios para poder establecer el verdadero potencial de esta técnica en el complejo infección/inflamación intraamniótica.
- De todos los metabolitos estudiados, el grupo de los esfingolípidos, especialmente esfingomielina y lactosilceramidas fueron los metabolitos que diferenciaban aquellas pacientes con corioamnionitis.
- La infección intraamniótica por Ureaplasma en pacientes con APP o RPM pretérmino no está asociada en todos los casos con una inflamación histológica. Aproximadamente el 40% no desarrollará ningún tipo de lesión.
- A la hora de identificar aquellos casos que desarrollarán corioamnionitis, IL 6, IL 8, IL 12 y MMP 8 podrían considerarse marcadores diagnósticos delesión.
- Valores de IL 6 en líquido amniótico por encima de 400 pg/ml detectó el 100% de los casos de corioamnionitis con una especificidad del 66%.

1. INTRODUCCIÓN

1.1 PARTO PRETERMINO

El parto pretérmino se define como aquel que ocurre antes de la semana 37 de gestación. A pesar de los grandes avances en la medicina intensiva neonatal, el parto pretérmino continúa siendo una importante causa de morbi-mortalidad neonatal. Aproximadamente uno de cada cinco niños con retraso mental y casi la mitad con parálisis cerebral serán debidos a la prematuridad [1].

La incidencia del parto por debajo de la semana 37 oscila entre el 7.6 y el 12 % de todos los nacimientos en los países desarrollados y de hasta el 15% de los nacimientos en los países en vías de desarrollo[2,3].

El parto pretérmino podemos subdividirlo a su vez en 4 grupos en función de la edad gestacional:

- Pretermino tardío: aquel que acontece entre la semana 34 y 37 de gestación. Representa la mayoría de los partos pretérminos, entre un 60-70% de ellos.
- Moderadamente pretérmino: aquel que acontece entre la semana 32 y 34 de gestación. Representa aproximadamente el 20% de los partos prematuros.
- Prematuridad severa: aquel que acontece entre la semana 28 y 32 de gestación. Representa el 15% de lospretérminos.
- Pretérmino extremo: aquel que acontece por debajo de la semana 28. El más severo y menos frecuente, constituyendo un 5% del total.

Entendemos por rotura prematura de membranas (RPM), la pérdida de integridad de las membranas ovulares antes del inicio del parto, con la consiguiente salida de líquido amniótico (LA) y la puesta en comunicación de la cavidad amniótica con el canal endocervical y la vagina [4]. La amenaza de parto pretérmino (APP) es el proceso clínico sintomático que sin tratamiento, o cuando este fracasa, podría conducir a un parto antes de las 37 semanas de gestación[5].

Tanto la RPM como la APP son dos entidades clínicas que confluyen en el parto pretérmino.

Aproximadamente, el 30% de todos los partos pretérminos son electivos o iatrogénicos, es decir, gestaciones finalizadas por indicaciones maternas o fetales.

Dentro de los que se producen de manera espontánea aproximadamente el 25% son secundarios a una RPM [6].

La etiología del parto pretérmino espontáneo es multifactorial. En los últimos años existe una tendencia a tratar esta entidad clínica como un síndrome, con múltiples mecanismos causales entre los que se incluyen la infección y una respuesta inflamatoria a ésta, la isquemia uteroplacentaria, la sobredistensión uterina, la insuficiencia cervical y otros procesos mediados inmunológicamente [7].

En este trabajo se estudiará de manera específica la causa infecciosa/inflamatoria del parto pretérmino.

1.2 INFECCIÓN INTRAAMNIÓTICA

En condiciones normales la cavidad amniótica es un medio estéril, libre de microorganismos cultivables o detectables mediante técnicas de microbiología molecular. Existen 4 vías por las que los microorganismos pueden colonizar la cavidad y causar una infección intraamniótica (IIA) [8-13]:

- Vía ascendente desde el tracto genital inferior.
- Vía hematógena en gestantes con bacteriemia.
- Vía accidental fundamentalmente por procesos invasivos como amniocentesis, cordocentesis o fetoscopias.
- Vía retrógrada desde cavidad peritoneal desde las trompas de Falopio, aunque existe evidencia contradictoria acerca de ésta última vía.

La invasión intraamniótica ascendente desde el tracto genital inferior es la vía más frecuente para el desarrollo de una IIA. Sin embargo, aunque todas gestantes presentan microorganismos en el tracto genital, la mayoría de ellas no desarrollan una IIA. El canal cervical, con el tapón mucoso, representa una barrera anatómica y funcional que impide la colonización intraamniótica [14-15].

La invasión microbiana de la cavidad amniótica podemos dividirla en 4 estadíos o etapas [16,17]:

- Estadío I: Alteración de la flora vaginal. Se produce un cambio en la flora cervical que permite la proliferación excesiva de microorganismos.
- Esstadio II: Disolución del tapón mucoso y acceso a la cavidad. En función de la virulencia y el tamaño del inóculo de estos gérmenes se producen enzimas proteolíticas que disuelven el tapón mucoso, invadiendo el espacio coriodecidual y provocando una reacción inflamatoria local.
- Estadío III: Infección de las membranas fetales. Los microorganismos pueden invadir los vasos fetales causando coriovasculitis o invadir el amnion causando una IIA. Estos microorganismos pueden atravesar las membranas intactas.
- Estadío IV: Infección fetal. La infección del feto se puede producir a través de la deglución del LA contaminado o por contacto directo con mucosas. La siembra de cualquiera de estos sitios a la circulación fetal puede resultar en bacteriemia y sepsis fetal.

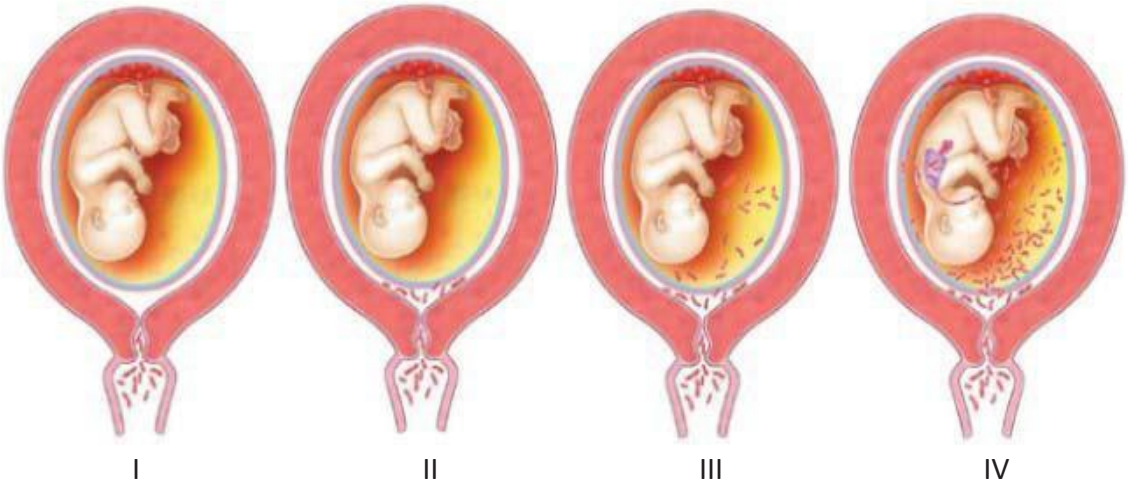

Figura 1. Etapas de la infección intraamniótica por vía ascendente. Adaptado de *Kim CJ. Am J Obstet Gynecol. 2015.*

A lo largo de los últimos años se ha documentado la presencia de IIA en pacientes con APP y membranas íntegras, RPM, incompetencia cervical, placenta previa, sangrado vaginal de origen desconocido y corioamnionitis clínica [16]. La RPM no es necesaria para el paso de microorganismos a la cavidad amniótica, de hecho, se sabe que pueden atravesar membranas íntegras [18]. La mayoría de estas infecciones son

asintomáticas y pasarán inadvertidas a menos que se analice el LA [19-21].Sin embargo, la evidencia actual apunta a la IIA como una de las principales causas de parto pretérmino siendo responsable en un 10-20% de los casos de parto pretérmino con membranas íntegras y de hasta un 30% de las RPM pretérmino[7,11,21,22].

La IIA no sólo es responsable del parto pretérmino sino también de, complicaciones neonatales y morbilidad materna como hemorragia postparto, endometritis postparto y corioamnionitis clínica [23,24]. Esta corioamnionitis clínica se caracteriza por la presencia de [25]:

- Fiebre materna y dos o más de los siguientescriterios:
- Taquicardia materna (> 100lpm)
- Taquicardia fetal (> 160lpm)
- Irritabilidad uterina
- Líquido amnióticopurulento
- Leucocitosis materna (> 15000-18000celulas/mm3)

Sin embargo este cuadro clínico ocurre únicamente en aproximadamente un 12.5% de la pacientes con parto pretérmino y bolsa íntergra [26] y refleja la manifestación sistémica materna a la invasión microbiana.

1 .2.1 *MICROBIOLOGÍA DE LA IIA*

Los microorganismos causantes de una bacteriemia materna pueden colonizar la cavidad amniótica a través de la vía hematógena [16]. Gérmenes como *Listeria monocytogenes*, *Treponema pallidum, Yersinia pestis*, Cytomegalovirus, *Plasmodium species*, y otros pueden acceder al espacio intervelloso a través de la circulación materna y desde allí acceder a la circulación fetal [12]. Bacterias causantes de la enfermedad periodontal suelen acceder a la cavidad amniótica siguiendo también esta vía [27].

Sin embargo los gérmenes más comúnmente encontrados en la cavidad amniótica son los mycoplasmas genitales, en particular *Ureaplasma* especies [28-36], *Gardnerella vaginalis* [19,37], *Fusobacteria* species, etc. [38]. Ciertos hongos también pueden ser los responsables de la IIA, fundamentalmente las mujeres embarazas portadoras de

dispositivos intrauterinos tienen un alto riesgo de desarrollar infección por *Candida albicans* [39].

Hasta en un 30% de los casos existe una invasión polimicrobiana de la cavidad amniótica [30,38,40,41].

1.2.2 INFECCIÓN POR UREAPLASMAUREALYTICUM

Una mención aparte merece la infección e inflamación intrauterina causada por Ureaplasma urealyticum.

Este germen es la bacteria más comúnmente aislada en líquido amniótico en relación con RPM y /o APP. Hasta un 33% de las IIA serán causadas por Ureaplasma [42,43].

Cuanto más temprana se produzca la infección, más probabilidades existen de aislar este microorganismo en el líquido amniótico, la placenta o las membranas amnióticas [43].

Sin embargo, Ureaplasma puede llegar a colonizar el espacio coriodecidual y el líquido amniótico sin llegar a desencadenar un proceso inflamatorio. De tal manera que según algunos trabajos [34,35] entre aquellas pacientes en las se evidencia una IIA por Ureaplasma urealyticum, menos del 25% dará lugar a un parto pretérmino, consiguiéndose así, gestaciones con una evolución favorable hasta el término en la mayoría de los casos. Este fenómeno puede ser explicado por diferentes motivos, por ejemplo, las propiedades inmunosupresoras de la placenta junto con la baja virulencia del microorganismo. Podemos especular que pequeños inóculos de Ureaplasma puede ser insuficientes para para inducir inflamación en un ambiente con elevada capacidad anti inflamatoria como la decidua [44].

Por lo tanto, las consecuencias clínicas de la presencia de Ureaplasma en el líquido amniótico y el desarrollo de una reacción inflamatoria intrauterina parece ser dosis y edad gestacional dependiente [45,46].

Microorganismos más frecuentes en pacientes con parto pretérmino	**Microorganismos más frecuentes en pacientes con corioamnionitis a término**
Fusibacterium nucleatum	*Ureaplasma species*
Sneathia sanguinegens	*Gardnerella vaginalis*
Ureaplasma species	*Mycoplasma hominis*
Streptococcus mitis	*Streptococcus agalactiae*
Gardnerella vaginalis	*Lactobacillis species*
Peptostococcus especies	*Bacterioides species*
Leptotrichia amnionii	*Acitenobacter species*
Mycoplasma hominis	*Sneathia*
Streptococcus agalactiae	*Streptococcus viridans*
Lactobacillis species	*Porphyromonas species*
Bacillus species	*Veillonella species*
Coagulase-negativo Staphylococcus species	*Peptostreptococcus species*
Prevotella species	*Escherichia coli*
	Pseudomona aeruginosa
	Staphylococcus aureus
	Enterococcus species
	Gram (-) bacilli

Tabla 1. Diferencias de microorganismos según el cuadro clínico. Adaptado de *Kim CJ. Am J Obstet Gynecol. 2015.*

1.2.3 PREVENCIÓN DE LA INFECCIÓNINTRAAMNIÓTICA

Las principales situaciones de riesgo identificadas hasta la fecha para el desarrollo de una IIA son:

- La vaginosisbacteriana
- La colonización vaginal por *Trichomonas vaginalis*
- La bacteriuria asintomática.

No está clara la prevalencia de vaginosis bacteriana, describiéndose grandes variaciones según la población estudiada. En población española se ha descrito hasta una prevalencia del 7.5% [47]. Por tanto la utilidad de realizar un cribado universal en toda la población está muy debatida. En el momento actual, no se considera beneficioso su aplicación en el general de la población gestante, aunque sí parece existir un beneficio en aplicar el cribado en población de alto riesgo, como aquellas gestantes con antecedentes de APP y/o RPM [48].

Del mismo modo, aunque se ha descrito la colonización por *Trichomonas vaginalis* como factor de riesgo para IIA, diferentes estudios han fracasado a la hora de demostrar una reducción del parto pretérmino con el tratamiento de este tipo de infecciones en mujeres asintomáticas [11,49]. Otro tipo de infecciones vaginales como por ejemplo la colonización por *Mycoplasmas* o *Candidas,* no han demostrado ser un claro factor de riesgo para parto pretérmino [50].

Hasta el momento, la única medida que ha demostrado tener un efecto beneficioso en la prevención del parto pertérmino es el tratamiento de la bacteriuria asintomática en la gestante [51].

1.2.4 DIAGNÓSTICO DE LA INFECCIÓNINTRAAMNIÓTICA

El diagnóstico de la IIA subclínica sólo se puede llevan a cabo con el análisis de LA mediante la realización de una amniocentesis diagnóstica.

Una vez analizado el LA se pueden obtener datos directos e indirectos acerca de la presencia de IIA.

Los **datos directos** serian: la positividad de test de PCR (reacción en cadena de la polimerasa), cultivo microbiológico positivo del LA o la visualización de gérmenes en la tinción de Gram. La positividad de estos test supone la presencia de gérmenes en LA. En los últimos años el desarrollo de técnicas de biología molecular para detectar material genético bacteriano mediante la técnica de PCR ha supuesto un gran avance, aumentando las tasas de detección dado, que no se necesitan grandes cantidades de gérmenes y no está falseado por la introducción de antibiótico antes de realizar la prueba. De esta manera la técnica de PCR parece haber desplazado a pruebas más clásicas como el cultivo microbiológico o la tinción de Gram en las que se necesita un

gran inóculo de germen para su detección y que por lo tanto ven más comprometida su sensibilidad.

En cuanto a los **datos indirectos** pasarían por la identificación de unos niveles disminuidos de glucosa en LA fruto del consumo por parte de los gérmenes, el aumento de los leucocitos como expresión de la inflamación tras la IIA.

La utilización de la amniocentesis diagnóstica tiene el potencial de detectar la infección subclínica antes de que se desencadene la cascada de fenómenos inflamatorios que están asociados con mayores complicaciones fetales, neonatales y maternas [52-54]. Sin embargo, la amniocentesis no deja de ser una prueba invasiva, que aunque tasas de complicaciones bajas, se ha asociado con RPM, parto pretérmino y hemorragia fetomaterna entre otras [55,56]. Además el hecho de diagnosticar mediante amniocentesis y potencialmente tratar esta IIA no ha demostrado reducir las tasas de prematuridad ni mejorar los resultados perinatales [57]. Por todo ello el uso sistemático de amniocentesis diagnóstica en pacientes con sospecha de IIA es aún controvertido y actualmente parece restringirse en la mayoría de los centros a fines de investigación.

1.2.5 TRATAMIENTO DE LA INFECCIÓNINTRAAMNIÓTICA

La invasión de la cavidad amniótica ha sido tradicionalmente atribuida a la presencia de bacterias en suspensión en el LA. Sin embargo, según estudios recientes [58-65] las bacterias pueden formar estructuras tipo “biofilms” en la cavidad amniótica. Estos biofilms son como estructuras organizadas de diferentes microorganismos adheridas a un substrato o bien a sí mismas. Se postula que la presencia de estos biofilms pueden ser clínicamente sospechada cuando observamos “sludge” en forma de partículas durante el examen ecográfico [58-65].

Figura 2. Presencia de "Sludge" durante examen ecográfico en paciente con APP.

Estas bacterias en biofilms están envueltas en una matriz extracelular de sustancias poliméricas y muestran un fenotipo diferente en cuanto a tasas de crecimiento y transcripción génica en comparación con las bacterias que se encuentran simplemente en suspensión en un medio. La mayoría de las bacterias incluidas en biofilms pueden crecer en un gran número de superficies distintas [66]. Estos biofilms son responsables de muchas infecciones humanas tales como la periodontitis o la endocarditis y son importantes dado que los gérmenes incluidos en biofilms son resistentes al tratamiento antibiótico. La formación de estas estructuras en el LA puede explicar la dificultad que existe a la hora de tratar la IIA.

Ciertos estudios señalan la posibilidad de erradicar la IIA diagnosticada mediante amniocentesis en pacientes con RPM pretérmino [67] y aquellas con cérvix corto asintomático [68] administrando antibiótico intravenoso. Estos estudios argumentan que la efectividad del tratamiento se debe a la detección precoz del cuadro, antes de que se desencadene la cascada de fenómenos inflamatorios que suele seguir a la IIA. Sin embargo este punto continúa siendo controvertido, no existiendo aún consenso en la pauta antibiótica ni conducta a seguir en estos casos en los que se sospecha IIA.

Probablemente, y dada la evidencia actual, la IIA sea curable en estadíos muy precoces y siempre y cuando el microorganismo patógeno presente una virulencia baja. Ciertos estudios han demostrado la eficacia del tratamiento antibiótico en IIA causada por gérmenes de baja virulencia como *Ureaplasma urealiticum* [68,69], sin embargo la mayoría de los intentos de tratamiento de IIA causados por gérmenes de alta virulencia como el *Fusobacterium nucleatum* ha fracasado hasta la fecha [68].

1.3 INFLAMACIÓNINTRAAMNIÓTICA

1.3.1 FISIOPATOLOGÍA

La placenta está formada por la fusión de las membranas fetales y la mucosa uterina o decidua. La decidua se considera de origen materno mientras que membranas amnióticas y las vellosidades coroideas son de origen fetal.

Los procesos inflamatorios que tienen lugar en la placenta se caracterizan por la infiltración de neutrófilos en estas estructuras. Por lo tanto, el origen del proceso inflamatorio puede determinarse examinando el origen de esta infiltración neutrófila; que podrá desarrollarse desde el compartimento materno generando una respuesta inflamatoria materna o bien fetal con el consiguiente síndrome de respuesta inflamatoria fetal (SRIF) [70].

Los neutrófilos no se encuentran en condiciones normales en las membranas corioamnióticas, sin embargo una vez los microorganismos colonizan la vagina, disuelven el tapón mucoso y generan una IIA , esta infección provoca la liberación de citoquinas y mediadores inflamatorios que provocan que los neutrófilos maternos que normalmente están presenten en el espacio intervelloso migren hacia el espacio coriodecidual. Cuando el proceso inflamatorio llega a afectan al corion y al amnios se denomina corioamnionitis aguda, y en este caso la respuesta inflamatoria será de origen materno considerándose una respuesta inflamatoria materna a la infección y puede desencadenar contracciones miometriales y RPM que desemboquen en un parto pretérmino [71].

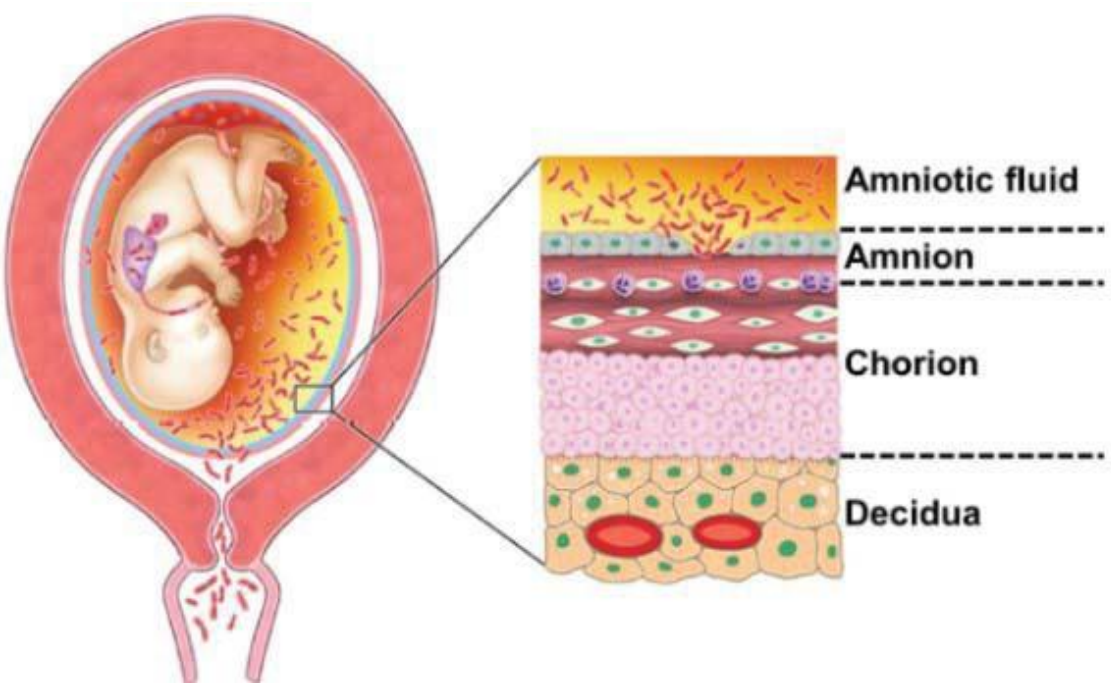

Figura 3. La infección intraamniótica puede progresar a una infección fetal e invasión del espacio coriodecidual. La destrucción del epitelio amniótico es uno de los rasgos característicos de la corioamnionitis. Adaptado de *Kim CJ. Am J Obstet Gynecol. 2015.*

Con los movimientos de respiración y deglución, el feto se expone a la colonización por parte de microorganismos que una vez entran en contacto con las mucosas fetales son reconocidos por diferentes sistemas de receptores del tipo Toll-like [72] induciendo de esta manera la producción de factores de transcripción y citoquinas proinflamatorias que generará la migración de neutrófilos desde la circulación fetal.

Los neutrófilos de origen fetal invaden las paredes de las arterias y la vena umbilical generando un proceso inflamatorio que culminará por la presencia de funisitis, la expresión histológica del SRIF. El gradiente que provoca la migración de neutrófilos desde el espacio luminal de los vasos fetales hacía las paredes y gelatina de Wharton está mediada por diferentes citoquinas y mediadores proinflamatorios presentes en el líquido amniótico y sangre fetal. De todas las citoquinas inflamatorias implicadas, son las concentraciones séricas de IL6 las que mejor que correlacionan con la intensidad y severidad del SRIF [73]. Redline [74] establece una clasificación por grados dentro de la

corioamnionitis histológica, diferenciando entre compartimento fetal y compartimento materno:

Corioamnionitis histológica **Compartimento materno**	**Corioamnionitis histológica** **Compartimento fetal**
Grado I: Subcorionitis/ corionitis aguda precoz. **Grado II**: Corioamnionitis aguda. **Grado III**: Corioamnionitis necrotizante. Severa: microabcesos corionicos. Prolongada: corioamnionitis crónica. Diagnóstico diferencial: Necrosis laminar. Corioamnionitis crónica no infecciosa.	**Grado I**: Flebitis umbilical / vasculitis coriónica. **Grado II**: arteritis umbilical. **Grado III**: periflebitis concéntrica. Severa : Intensa vasculitis coriónica. Prolongada: Funisitis necrotizante. Diagnóstico diferencial: Vasculitis por eosinófilos T Villitis de etiología desconocida con vasculopatía obliterante fetal.

Tabla 2. Clasificación histológica de la corioamnionitis. Adaptado de *Redline RW. Semin Fetal Neonatal Med. 2012.*

Si bien estos cambios histológicos pueden ser consecuencia de agresiones no infecciosas, como daño hipóxico, meconio, alérgenos etc, una infección intraamniótica es la causa más común. Según Lahra [75] la corioamnionitis histológica está presente en el 60-80% de las placentas de los partos menores de 28 semanas, 40-50% de las placentas de los partos de 29 a 34 semanas, y en el 5-30% de los partos por encima de las 34 semanas.

Actualmente se considera que la inflamación intraamniótica está presente en aproximadamente 10-30% de las pacientes con APP [21,76] y en el 40% de las RPM [77] y se considera el mayor predictor de infección intraamniótica.

1.3.2 SINDROME DE RESPUESTA INFLAMATORIAFETAL

Gómez y colaboradores [78] definieron por primera vez el término SRIF para aquel proceso inflamatorio en el compartimento fetal de la gestación caracterizado por una elevación de la concentración de IL 6 en sangre fetal (IL 6 > 11 ng/ml). En el estudio realizado por Gómez et al demostraron que los neonatos en los que lo valores de IL6 en sangre fetal fueron mayores de 11 ng/ml tenían mayores tasas de morbilidad fetal. La funisitis, definida como la infiltración perivascular de células inflamatorias en los vasos umbilicales, se considera uno de los mayores predictores de SRIF [79-81].

El SRIF se asocia a un fallo multisistémico fetal e influye la afectación del sistema nervioso central (SNC), sistema hematopoyético y cardiaco, sistema respiratorio y sepsis fetal entre otros. Debido a la elevación de citoquinas inflamatorias en sangre fetal y líquido amniótico se pueden llegar a desarrollar una serie de secuelas fetales y neonatales como la leucomalacia periventricular (LPV), parálisis cerebral, displasia broncopulmonar (DBP), sepsis fetal y disfunción cardiaca fetal [82-84].

De tal manera podemos concluir que la expresión analítica del SRIF pasaría por una elevación de la IL6 en sangre fetal; la expresión histológica es la presencia de neutrófilos en los vasos umbilicales o funisitis y finalmente la expresión clínica es un aumento de la moribimortalidad fetal.

1.3.3 CONSECUENCIAS NEONATALES

La IIA y el consiguiente desencadenante de SRIF puede ser la causa de una encefalopatía hipóxico–isquémica debido a la producción de citoquinas fetales que atraviesan la barrera hematoencefálica y facilitan la entrada de productos microbianos y citoquinas al cerebro [79].

Estas citoquinas tienen un efecto citolítico sobre las neuronas y los precursores de oligodendrocitos que se harán sensibles a la isquemia provocando una desmielinización de la sustancia blanca. Las citoquinas también se han relacionado con otros efectos neutotóxicos como la liberación de amniocitos excitadores, la inducción de la apoptosis, la alteración de la cascada de la coagulación e hipotensión fetal.

Esta encefalopatía hipoxico isquémica va a producir una necrosis de la sustancia blanca en la región periventricular produciendo un cuadro de atrofia, ventriculomegalia y formación de quistes, dando lugar al cuadro clínico conocido como leucomalacia periventricular que puede ser la causa de parálisis cerebral en el neonato [85].

El SRIF también se ha relacionado con la generación intraútero de disfunción cardiaca fetal. El mecanismo a través del cual se produce esta disfunción, vendría determinado una vez más por el aumento de las citoquinas proinflamatorias en sangre fetal. La liberación de estos mediadores inflamatorios da lugar a un aumento en la distensibilidad del ventrículo izquierdo, desembocando en disfunción cardiaca fetal [84,86,87]. Además, en estados de sepsis fetal severa la depresión miocárdica puede conducir a la muerte fetal.

La presencia de corioamnionitis histológica aumenta también las probabilidades de encontrar en los neonatos marcadores de infección, colonización bacteriana y sepsis congénita [88]. De hecho aproximadamente un tercio de las pacientes con IIA tienen una bacteriemia fetal [24]. Un estudio encontró mayores tasas de neutrofilia, proteína C reactiva elevada y abundancia de leucocitos en el lavado gástrico de los neonatos en los que se demostró una corioamnionitis histológica [88].

1.4 CITOQUINAS PARA EL ESTUDIO DE LA INFECCIONINTRAAMNIÓTICA

La invasión microbiana de la cavidad amniótica induce una respuesta inflamatoria local que se acompaña de una liberación de citoquinas pro-inflamatorias como IL 1b [89,90], factor de necrosis tumoral alfa (TNF-a) [82,91], IL 8 [82,91], IL 6 [92,93] entre otros.

La concentración de citoquinas, metaloproteasas y otros productos liberados durante el curso del proceso inflamatorio han sido estudiados a lo largo de los últimos años para determinar su papel en el diagnóstico y pronóstico en casos de sospecha de infección/inflamación intraamniótica. Hasta el momento las concentraciones en líquido amniótico de IL 6 y MMP 8 parecen ser los mejores predictores de morbilidad neonatal en pacientes con parto pretermino con membranas íntegras [38,94], RPM[40,77].

La IIA en pacientes con APP o RPM se ha asociado de manera significativa a una elevación de IL6 en líquido amniótico [78]. Determinadas citoquinas inflamatorias tales como la IL1b y TNF-a producen una liberación de IL6 en el corion y las células deciduales. Esta IL6 está relacionada con la inducción de la síntesis de la proteína C reactiva y con la liberación de prostaglandinas que pueden llevar a la producción de contracciones uterinas y modificación cervical con rotura de membranas.

La IL6 es la citoquina más probablemente relacionada con la fisiología del parto pretermino, la IIA y la corioamnionitis histológica [95-98] siendo hasta la fecha el marcador más sensible de IIA con una sensibilidad de en torno al 80- 100% [99].

	SENSIBILIDAD	ESPECIFICIDAD	VPP	VPN
IL 6 > 11 MG/DL	100%	82.5%	36.7%	100%

Tabla 3. Valores predictivos de IL6 para el diagnóstico de infección intramniótica. Adaptado de *Romero R, Am J Obstet Gynecol. 1993.*

La MMP 8 o Metaloproteasa- 8 es una enzima de la familia de las colagenasas que es producida por los neutrófilos de origen fetal presentes en el líquido amniótico durante la respuesta inflamatoria a una IIA [100].

Por lo tanto una elevación en la MMP 8 podría indicar una respuesta inflamatoria de origen fetal. En los últimos años ha surgido un gran número de estudios apuntando a la MMP 8 como un importante marcador de infección/inflamación intraamniótica [101-104] e incluso el grupo de Romero [105] ha patentado un test de detección de rápida de MMP8 en líquido amniótico para la detección de inflamación intraamniótica en casos de sospecha de IIA como APP o RPM con altas tasas de sensibilidad y especificidad. Sin embargo, el uso de este test de detección rápida de MMP 8 está lejos de ser implementado por el momento en la práctica clínica diaria en nuestro medio.

CITOQUINAS	*FUNCIÓN*
IL - 1 a	Molécula endógena indicadora de daño celular y tisular Efecto proinflamatorio produciendo la liberación de más citoquinas Mediador del reclutamiento neutrofílico
IL- 1b	Importante mediador de la respuesta inflamatoria
IL - 6	Mediador clave en la fase aguda de respuesta a la infección Activador de las células T y las celular *Natural Killer* Estimula la proliferación de células B y la producción de inmunoglobulinas
TNF - a	Importante mediador de la sepsis
IL - 4	Citoquina anti-inflamatoria Inhibe producción de ILb Induce la diferenciación de células T Estimula la producción de Ig G e Ig E
IL - 10	Citoquina anti-inflamatoria Inhibe la producción de citoquinas proinflamatoria Supresor de macrófagos y células *Natural Killer*
IL - 8	Reclutamiento y activación de células de inflamación aguda como neutrófilos Promueve la angiogénesis

Tabla 4. Algunos de los más importantes mediadores pro y anti inflamatorios que participan en el proceso de inflamación intrauterina. Adaptado de *Kim CJ. Am J Obstet Gynecol. 2015*

1.5 METABOLÓMICA PARA EL ESTUDIO DE LA INFECCIÓNINTRAAMNIÓTICA.

La infección perinatal parece jugar un papel fundamental en la patogénesis de daño neuronal en los neonatos pretérmino. El desarrollo de este daño neuronal está causado en parte por una respuesta inflamatoria mediada por una elevación de citoquinas proinflamatorias en el líquido amniótico y cordón umbilical [82,106].

Diferentes biomoléculas juegan un papel fundamental en este proceso, promoviendo o bien protegiendo este proceso de daño cerebral en el neonato pretérmino. La investigación de estas biomoléculas podría ayudar a encontrar nuevos marcadores que puedan predecir o ayudarnos a entender mejor la fisiopatología de estos procesos.

La proteómica y la metabolómica son dos nuevas y prometedoras tecnologías que estudian las secuencias fenotípicas en procesos como el daño neuronal. Son herramientas complementarias al estudio genómico pero que en vez de basarse en la identificación del gen responsable, estudian las proteínas y metabolitos que se expresan en una determinada patología y que serán las que, en realidad, generan el cuadro patológico.

Ambas, tanto proteómica como metabolómica, revelan las diferentes interacciones entre la madre, la placenta y el feto. Permitiéndonos así identificar elementos para el diagnóstico y la evolución de la corioamnionitis y lesiones asociadas como el daño neuronal.

Existen algunos estudios que intentan evaluar el uso potencial de algunas proteínas cerebrales como la S100B, enolasa y la proteína glial fibrilar, sin embargo, estos estudios se centran en el daño hipóxico cerebral y no en el potencial proceso inflamatorio desencadenante [83, 107].

Existen pocos trabajos hasta la fecha que estudien secuencias metabolómicas en líquido amniótico. Algunos de ellos han estudiado los mecanismos responsables del parto pretérmino y su predicción. Sin embargo, en una reciente revisión [108] tan sólo se identificaron 6 estudios centrados en la metabolomica del líquido amnióticos y ninguno de ellos estudiaba de manera específica la patología de la corioamnionitis o los mecanismos fisiopatológicos del daño neuronal perinatal.

El estudio de estos procesos a nivel molecular es un reto importante a la hora de entender mejor la fisiopatología del proceso inflamatorio intrauterino y poder así obtener secuencias metabolómicas para la identificación de biomarcadores que puedan ser utilizados como una nueva estrategiadiagnóstica.

2. HIPÓTESIS DE TRABAJO.

La hipótesis general de este estudio es que pueden encontrarse modelos multivariantes predictivos que utilizan tanto parámetros clínicos como conocidos y nuevos marcadores bioquímicos en el líquido amniótico obtenido mediante amniocentesis para predecir la presencia de infección intrauterina, su grado de severidad y su potencial repercusión feto-neonatal. Esta hipótesis general se desarrolla en las siguientes hipótesis específicas:

1. Una combinación de parámetros clínicos y marcadores bioquímicos de inflamación medidos en el líquido amniótico puede identificar los casos de corioamnionitis y funisitis histológica y sus grados de severidad.

2. El estudio metabolómico del líquido amniótico en casos de sospecha de infección intraamniótica puede ayudar a encontrar nuevos biomarcadores que sirvan para identificar aquellos casos de corioamnionitis histológica, sus diferentes grados de severidad así como para la predicción de la morbilidad neonatal asociada a este proceso, especialmente el daño neurológico.

3. La infección intraamniótica por Ureaplasma urealyticum se relaciona con una menor severidad del proceso inflamatorio intrauterino y podría existir una combinación de parámetros bioquímicos medidos en el líquido amniótico que nos ayuden a identificar la presencia de corioamnionitis histológica en estas pacientes.

3. OBJETIVOS.

1. Identificar una combinación de citoquinas inflamatorias capaces de diferenciar entre la presencia de corioamninonitis y corioamninitis más funsitis, en aquellas pacientes con alto riesgo de infección intraamniótica. (Estudio 1).

2. Identificar biomarcadores con el estudio metabólico del líquido amniótico en pacientes con alto riesgo de infección intraamniótica, capaces de identificar aquellas pacientes con corioamnionitis (Estudio 2).

3. Identificar biomarcadores con el estudio metabólico del líquido amniótico en pacientes con alto riesgo de infección intraamniótica, capaces de predecir lesión neurológica perinatal (Estudio2).

4. Determinar la frecuencia de corioamnionitis y de corioamninitis más funisitis en aquellas pacientes con infección intraamniótica por *Ureaplasma urealyticum* (Estudio 3).

5. Determinar la capacidad predictiva de determinadas citoquinas inflamatorias para el diagnóstico de corioamnionitis en casos de infección intraamniótica por *Ureaplasma urealyticum* (Estudio3).

6. Analizar el potencial impacto de la terapia con Azitromicina en la prevención de inflamación histológica en casos de infección intraamniótica por *Ureaplasma urealyticum* (Estudio 3).

4. INVESTIGACIONES REALIZADAS. METODOLOGÍA Y RESULTADOS GLOBALES.

4.1 METODOLOGÍA DEL ESTUDIO

Los tres estudios que conforman la presente tesis forman parte de una misma línea de investigación basada en la identificación de biomarcadores en líquido amniótico capaces de predecir y diferenciar entre los diferentes estadíos de inflamación intrauterina. El diseño del estudio, la población analizada así como la metodología utilizada se detallan en los apartados de "Material y Métodos" de cada uno de los artículos que constituyen el cuerpo doctrinal de la presente Tesis Doctoral.

Dichos artículos se incluyen a continuación tal y como han sido aceptados para publicación en la literatura científica.

4.2 INVESTIGACIONES REALIZADAS

ESTUDIO 1

Differential amniotic fluid cytokine profile in women with chorioamnionitis with and without funisitis. Revello R, Alcaide MJ, Dudzik D, Abehsera D, Bartha JL. J Matern Fetal Neonatal Med. 2016;29(13):2161-5

ESTUDIO 2

LC-MS-based metabolomics identification of novel biomarkers of chorioamnionitis and its associated perinatal neurological damage. Dudzik D, Revello R, Barbas C, Bartha JL. J Proteome Res. 2015 Mar 6;14(3):1432-44.

ESTUDIO 3

Prediction of Chorioamnionitis in Cases of Intraamniotic Infection by Ureaplasma Urealyticum in Women with Very Preterm Premature Rupture of Membranes or Preterm Labour. Revello R, Alcaide MJ, Abehsera D, Martin-Camean M, Sousa E Faro Gomes M, Alonso-Luque B, Bartha JL. J Matern Fetal Neonatal Med. 2017May14:1-13.

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ORIGINAL ARTICLE

Differential amniotic fluid cytokine profile in women with chorioamnionitis with and without funisitis

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Abstract

Objectives: To evaluate whether the amniotic fluid (AF) cytokine profile in women with chorioamnionitis may differentiate between those with and without funisitis.
Subjects and methods: Forty women at high risk of chorioamnionitis were studied. Gestational age at study was 26.94. Amniocentesis, universal and specific polymerase chain reaction, and microbiological cultures were performed. AF IL-1b, IL-2, IL-4, IL-6, IL 8, IL-10, IL-12, TNF-alpha, IFN-gamma, and MMP-8 were measured by multiplex assay. After delivery, the placenta and umbilical cord were studied histologically. Comparisons were made between three groups: controls, and chorioamnionitis with and without funisitis.
Results: In 25 cases, the histological findings were normal (61.5%). The remaining 15 composed of 9 cases of chorioamnionitis alone (9/40; 23.1%) and 6 cases of chorioamnionitis plus funisitis (6/40; 15.4%). All AF cytokine levels were significantly higher in the cases with chorioamnionitis in comparison to controls, except for IFN-gamma. The comparisons between the three groups showed significant differences between chorioamnionitis alone and chorioamnionitis plus funisitis in IL-1b, IL-6, IL-10, IL-12, IL-8, and TNF-alpha, with the levels being higher when funisitis was present. Logistic regression found a powerful predictive model for funisitis including the following cytokinesIL-4, IL-10, IL-12, and IL-8.
Conclusions: Measurements of AF interleukins 4, 10, 12, and 8 allow to identify cases with funisitisin women at high risk of chorioamnionitis.

Keywords

Chorioamnionitis, cytokines, funisitis, inflammation

History

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Introduction

Preterm birth remains an unsolved problem in perinatal medicine. Its ethology is complex, but previous studies suggest that the infection–inflammation complex has a major weight in its origin, especially in spontaneous preterm deliveries at very early gestational ages [1,2]. It is estimated that approximately 60% of preterm deliveries are due to *in utero* infection [3].

The origin of this infection in the vast majority of cases is due to the ascent of microorganisms from the genital tract [4]. This intra-amniotic infection induces the release of cytokines and inflammatory mediators into the maternal circulation, and leads to inflammation of decidua and amniotic membranes. This can trigger myometrial contractions, membrane rupture, escalating into preterm labor [5].

With breathing and swallowing movements, the fetus is exposed to the entry of microorganisms into its circulation. These microorganisms provoke, in the same way as in the mother, a fetal inflammatory response with release of cytokines and inflammatory mediators. The inflammatory process can evolve to have systemic repercussions, causing lesions and fetal sepsis, a condition known as the fetal inflammatory response syndrome (FIRS) [3]. This syndrome is manifested histologically by the development of funisitis [6,7].

Thus, the inflammation that occurs in the setting of an intra-uterine infection vary in degree, and can progress to fetal sepsis [6–8]. Therefore, it specifies that intra-amniotic infection–inflammation complex is a variable phenomenon, probably with different degrees of inflammation that will escalate into different levels of both maternal and fetal morbidity and mortality [9–14].

It is essential, therefore, when evaluating the perinatal consequences of this condition to be able to differentiate between the different inflammatory states at intra-uterine level. Many studies strongly emphasize the relationship of inflammatory cytokines with preterm birth, adverse neonatal outcomes and also the presence of chorioamnionitis [5,10,15–20]. However, currently, there is no mediator or inflammatory molecule that allows to distinguish between pregnancies

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which have developed only chorioamnionitis and those in which inflammation has reached the fetal component.

The objective of the present study was to find a profile of amniotic fluid (AF) inflammatory cytokines that is diagnostic of the presence of chorioamnionitis and funisitis in high-risk patients. Therefore, the aim was to establish identifying parameters of those patients at risk of intra-amniotic infection who, in addition to develop chorioamnionitis, might evolve to inflammatory lesions at the fetal level. This could potentially be useful to offer a more specific prognosis of neonatal outcomes to guide decisions on what decision to take regarding the management of the pregnancy.

Material and methods

Forty pregnant women at high risk of preterm labor and chorioamnionitis were enrolled for the study. The inclusion criteria were: very preterm premature rupture of membranes (PPROM) (gestational age between 24 and 32 weeks) and/or preterm labor with poor prognosis (gestational age between 24 and 28 weeks, refractory to tocolysis, prolapsed amniotic sac in vagina, and/or vaginal bleeding from uterine origin of unknown cause). The exclusion criteria were: multiple gestations, the presence of clinical chorioamnionitis, and/or cases in which amniocentesis was clinically not recommended, if it was considered to be an additional risk factor for the course of the pregnancy.

PPROM was diagnosed by the presence of AF leakage in the vagina and/or by using a test detecting IGFBP-1 (insulin-like growth factor binding protein-1) in vaginal samples in the presence of olygohydramnios. Preterm labor was diagnosed by the presence of at least two regular uterine contractions every 10 min associated with cervical length ≤15 mm that required hospital admission and tocolytic treatment.

Patients meeting the inclusion criteria were offered amniocentesis to assess the microbial status of the amniotic cavity. An extra 3 mL of AF for the study was obtained after the patient gave her written informed consent. After amniocentesis, the AF was immediately transported in a capped sterile syringe to the biobank, and stored at −80 °C until analysis. The study was approved by the local Ethical and Research Committees, and all the women in the study signed a written consent form for their participation. All the samples were collected in a single institution between March 2011 and December 2013.

A molecular analysis was performed as follows: Total DNA was extracted from 400 mL of AF sample and eluted in 100 mL of elution buffer using a MagNA Pure Compact system and the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics GmbH, Mannheim, Germany). Detection of bacterial DNA was done by broad-range real-time polymerase chain reaction (PCR), targeting the 16S rRNA gene using Takara Premix Ex Taq and oligonucleotides P891F, P1033R, and the Taqman probe Uniprobe7. Specific assays were developed by substituting the forward primer by primers ChlaF 5′-GTATGCCGCCTGAGGAGTACA-3′ for Chlamydia sp., MychF 5′-GCCTGAGTAGTATGCTCGCA AGA-3′ for *Mycoplasma* sp., and UreF 5′-GCCTG GGTAGTACATTCGCAAGA-3′ for *Ureaplasma* sp. Each sample was tested in parallel with the universal and the three specific sets. Negative and positive controls were included in each assay. All the positive samples were identified by amplification and pyrosequencing of three short regions of the 16S rRNA gene as described elsewhere [20].

The AF processing for culturing aerobic and anaerobic bacteria was performed as follows: The sample was centrifuged, the supernatant discarded, and the product planted onto four different media. Gram staining was performed for the direct examination of Gram-positive bacteria. The growth media used were: blood agar (in a CO_2 incubator), chocolate agar (also in a CO_2 incubator), blood agar enriched with vitamin K1 and hemin (anaerobic incubation) for anaerobic microorganisms, and plating liquid (thioglycolate). The samples were evaluated every 24 h, except that, when the planting was performed anaerobically, the initial evaluation was at 48 h. The culture medium used for the detection of urogenital mycoplasmas is a commercial medium ''Mycofast Evolution 2'' (ELITech France SAS, France).

The gross examination of the placenta in the Pathology Department included: weight and other standard measurements; color, appearance, and integrity of membranes; description of any gross lesion; and length, color, insertion, coiling and vessels in the umbilical cord. The histological study included: three sections of cord (proximal, middle, and distal to the placenta); one membrane roll (by the ''jelly roll'' method in order to obtain a maximum amount of membranes with decidua capsularis); and three full thickness sections of parenchyma, including chorionic plate vessels. Tissues were fixed in 10% formalin solution for at least 6 h before paraffin embedding, followed by hematoxylin and eosin staining.

Histological chorioamnionitis and funisitis were classified according to the severity of the injury in the following specific pathologic patterns: (A) Chorioamnionitis: early acute subchorionitis/chorionitis, acute chorioamnionitis and necrotizing chorioamnionitis and (B) Funisitis: umbilical phlebitis/chorionic vasculitis, umbilical arteritis and concentric periphlebitis/necrotizing.

The cytokines TNF-alpha/IFN-gamma/IL-1b/IL-2/IL-6/IL-8/IL-12/IL-4/IL-10/MMP-8 were measured using a multiple immunoassay kit with magnetic beads (Immunoassay Kit Magnetic Plex Human 10, France) of Affymetrix, following the manufacturer's instructions.

The plates consisted of 96 wells, 16 used to perform the calibration curve from 8 standards processed in duplicate, 2 used for the blank, and the remaining 78 wells for samples of AF (25 mL) done in duplicate. The quantitative readout was conducted on a Luminex-200 xMap Technology® (France).

The results were analyzed using the SPSS 15.0 software package (SPSS, Chicago, IL). The distribution of variables was assessed by analyzing the histograms. Since most of the variables are distributed non-parametrically, the values are presented as median and interquartile range. Comparison between groups was performed using the Kruskal–Wallis' test. The *post-hoc* analysis comparing paired groups was performed with the DNS test. Prediction from cytokine data for the presence of funisitis was estimated by binary logistic regression analysis. The pre-set level of significance was 95% ($p \leq 0.05$).

Table 1. Amniotic fluid cytokine concentrations in the studied groups.

	Controls	Chorioamnionitis	Corioamniotitis + Funisitis	Diferences between groups p ¼	Analysis *post-hoc* p ¼
IL 1b (pg/mL)	2.83 (1.51–11.10)	14.62 (9.63–23.11)	34.43 (22.91–228)	50.0001	*0.211 y50.0001 **0.007
IL2 (pg/mL)	5.22 (2.21–10.33)	11.37 (8.18–14.78)	11.95 (8.74–19.61)	0.008	*0.019 y0.003 z0.375
IL4 (pg/mL)	25.45 (14.43 –51.50)	39.58 (31.55–63.12)	57.04 (48.36–81.47)	0.017	*0.258 y0.005 z0.097
IL6 (pg/mL)	64.65 (32.89–284.50)	866 (465–1105)	1244.5 (784.23–1686)	50.0001	*50.0001 y50.0001 z 0.011
IL10 (pg/mL)	8.31 (5.01–17.62)	32.22 (16.03–42.24)	88.03 (31.96–186.70)	50.0001	*0.124 y50.0001 z 0.008
IL12 (pg/mL)	35.36 (22.98–55.14)	94.78 (67.12–109.5)	140 (103.94–223)	50.0001	*50.0001 y50.0001 z 0.033
IL8 (pg/mL)	37.67 (17.67–79.77)	205 (142.50–346)	439.50 (170.75–581.5)	50.0001	*50.0001 y50.0001 z 0.0033
IFNg (pg/mL)	1.12 (0.53–2.67)	2.20 (1.40–3.45)	2.54 (1.42–4.53)	0.098	*0.368 y 0.129 z 0.509
TNFa (pg/mL)	6.63 (3.66–19.34)	19.98 (12.66–35.83)	48.14 (22.38–202)	0.01	*0.370 y 0.001 z 0.013
MMP-8 (pg/mL)	312 (187–545)	2305 (1343.5–4379)	3442.50 (937–7664.25)	50.0001	*0.001 y50.0001 z0.85

*Control versus chorioamnionitis.
yControl versus chorioamnionitis + funisitis.
zChorioamnionitis versus chorioamnionitis + funisitis.

Results

From the total of 40 women included in the study, 12 (30%) were cases of preterm labor with intact membranes and poor prognosis as described above and 28 (70%) were cases of PPROM. Maternal age was 32.63 ± 5.61 years; 27 were nulliparous (65.5%), and the mean gestational age at study was 26.91 ± 2.59 weeks.

In total, 18 patients (45%) were diagnosed of having an intra-amniotic infection. PCR was positive in all of them, whereas the microbiological culture showed infection in just 4 of them (4/40, 10%). The most commonly identified bacterium was *Ureaplasma urealyticum* (14/40, 35%).

After placental pathological study, we found 25 cases with no significant histological lesions (62.5%). Of these 25 cases without lesions, 19 had no diagnosis of intra-amniotic infection. Infection was diagnosed in the remaining 6 cases from identifying *U. urealyticum* by the AF PCR.

The 15 cases in which placental histological lesions were demonstrated, consisted of chorioamnionitis alone (9/40, 22.5%) and chorioamnionitis associated with funisitis (6/40, 15%). There was no case of funisitis without chorioamnionitis. Of these 15 cases with placental histological lesions, 12 had a prior diagnosis of intra-amniotic infection, and the other 3 were negative in both PCR and culture of the AF.

Of the 18 cases with intra-amniotic infection, 15 presented placental inflammatory alterations (15/18, 83.3%), although only 33% of the intra-amniotic infection cases developed funisitis.

The medians and interquartile ranges of the studied cytokines are listed in Table 1. There were statistically significant differences between the groups with and without histological inflammatory lesions for all the pro-inflammatory molecules studied except for IFN-gamma, for which the difference was borderline (p ¼ 0.05).

In the group-to-group comparison (*post-hoc* analysis), we performed three types of comparisons: (1) comparing cases of no histological lesions with cases of chorioamnionitis alone; (2) comparing cases of no histological lesions with cases of chorioamnionitis plus funisitis; and (3) comparing cases of chorioamnionitis alone with cases of chorioamnionitis plus funisitis. These three comparisons were made for each one of the studied cytokines.

Only for IL-6, IL-12, IL-8, and MMP-8, there were statistically significant differences between the controls and the chorioamnionitis alone groups. However, comparison between controls and the group with the chorioamnionitis plus funisitis group showed significant differences in most of the studied cytokines: IL-1b, IL-2, IL-6, IL-10, IL-12, IL-8, TNF-alpha, and MMP-8. For the comparison of chorioamnionitis alone with chorioamnionitis plus funisitis, there were significant differences in IL-1b, IL-6, IL-10, IL-12, IL-8, and TNF-alpha.

The binary logistic regression analysis gave a model for the prediction of funisitis with a high predictive power (R^2 ¼ 1, p 5 0.0001), which included in the equation the following cytokines: IL-4, IL-8, IL-10, and IL-12.

We have explored the ratios pro-inflammatory to anti-inflammatory cytokines among those selected by the prediction model. More specifically, we explored the predictive capability of IL8:IL10, IL8:IL4, IL12:IL10 and IL12:IL4 ratios. None of them were statistically significantly different between patients with or without chorioamnionitis (p ¼ 0.92, p ¼ 0.17, p ¼ 0.15 and p ¼ 0.28, respectively). None of them were included in the prediction model as independent variables.

Discussion

In recent years, the fundamental role of intra-amniotic inflammation in the development of preterm labor has become evident. However, it is the fetal systemic expression of these inflammatory processes which will lead to lesions that may end in fetal sepsis and multiple organ failure [21,22]. It should be considered, however, that not all intra-amniotic infections will lead to an inflammatory process extending to the fetal component. The results of the present study showed that, while 83% of the patients with suspected infection developed histological chorioamnionitis, only 33% progressed to funisitis or FIRS.

Although various recent studies [13,14] have defended the existence of increased neonatal morbidity in cases in which there is involvement of the fetal component in comparison with those of chorioamnionitis alone, there is still a lack of information that would allow us to catalog the different degrees of intra-uterine inflammation and their consequences for the newborn.

In the present study, we analyzed 10 inflammatory mediators related to a greater or lesser extent with the presence of intra-uterine infection. There were statistically significant differences between cases with no histological lesions and those with inflammatory lesions in all those molecules except IFN-gamma, for which the difference was borderline (p ¼ 0.05). Three of these 10 molecules, IL-6, IL-8, and IL-12, presented statistically significant differences in all three comparisons: control versus chorioamnionitis; control versus chorioamnionitis plus funisitis; and chorioamnionitis versus chorioamnionitis + funisitis. Thus, each of these cytokines is by itself capable to differentiate both the presence of inflammatory lesions in cases of intra-amniotic infection and the extension of the infection.

The relationship of IL-6 and IL-8 with FIRS is well known [23–27], but not that of IL-12. There have even been studies that have rejected any relationship of this molecule with preterm delivery in patients with suspected intra-uterine inflammation [27–29]. It has to be taken into account that not many works have explored the relationship between FIRS and IL-12, and most of them used small sample sizes. Also, these studies related the levels of interleukins with the results of the microbiological cultures rather than with pathological studies of the placenta. Finally, many of them focused on cytokine levels in media other than the AF, such as maternal blood or umbilical cord blood [27–29]. This could explain the differences with the present study, where we found AF IL-12 to be one of the most predictive of the 10 molecules studied in the diagnosis of FIRS.

In order to define a model or combination of cytokines that would predict the detection of funisitis in patients with intra-amniotic infection, we performed a logistic regression analysis that encompassed the 10 studied cytokines. The resulting formula included only four of them – IL-4, IL-8, IL-10, and IL-12 – which, surprisingly, were those which have been less studied up to now. The formula had a high predictive power (R^2 ¼ 1, p 5 0.0001). It appears that the combination of these four inflammatory mediators can detect practically all the funisitis cases among patients at risk with a greater precision than any of the isolated cytokine measurements, including some of those which have been extensively studied and validated such as IL-6 [16,24,25]. According to these findings, it seems that despite the fact that classic cytokines such as IL-6 are extremely elevated in cases of chorioamnionitis, they cannot distinguish between those with or without cord involvement.

This is a mathematical prediction model that includes two pro-inflammatory cytokines (IL-8 and IL-12) and two anti-inflammatory cytokines (IL-4 and IL-10). We can speculate that increased anti-inflammatory cytokines may have a role as a compensatory mechanism to dampen inflammation. A loss in the equilibrium between pro-inflammatory and anti-inflammatory cytokines may be important to put gestation at risk of develop FIRS.

In the same way as for IL-6, in recent years MMP-8 has emerged as one of the most specific molecules when establishing a fetal inflammatory process. It has been reported that it is capable of detecting cases of funisitis among patients at risk with a sensitivity close to 100% [11,12,15]. The present results are coherent with the published data, since statistically significant differences were found between the control group and the chorioamnionitis alone and chorioamnionitis plus funisitis groups. However, the present results do not allow considering MMP-8 as the most specific molecule for differentiating intra-uterine inflammatory events. Although it is a powerful marker for identifying funisitis among the overall population of patients at risk, we did not find differences in MMP-8 levels that would differentiate the two degrees of histological involvement – maternal and fetal– once established the intra-uterine inflammatory process.

Thus, although IL-6 and MMP-8 are two of the molecules that have been most studied and related to the diagnosis of intra-amniotic inflammation. In the future it might be the combination of several molecules rather than a single one, which could bring us closer to identify each of these stages of intra-uterine inflammation, and thus be able to provide an adequate prognosis for pregnant women with this condition. Further studies using a bigger number of patients should be done to affirm that the model has clinical value and to predict their capability in the presence of signs of neonatal infection.

Declaration of interest

The authors report no conflict of interest. This study was supported by a grant of the Institute of Health Carlos III (PI09/02179).

References

1. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet 2008;371:75–84.
2. Romero R, Espinoza J, Goncalves LF, et al. The role of inflammation and infection in preterm birth. Semin Reprod Med 2007;25:21–39.
3. Kuypers E, Ophelders D, Jellema RK, et al. White matter injury following fetal inflammatory response syndrome induced by chorioamnionitis and fetal sepsis: lessons from experimental ovine models. Early Hum Dev 2012;88:931–6.
4. Romero R, Gomez R, Ghezzi F, et al. A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. Am J Obstet Gynecol 1998;179:186–93.
5. Hagberg H, Mallard C, Jacobsson B. Role of cytokines in preterm labour and brain injury. BJOG 2005;12:16–18.
6. Pacora P, Chaiworapongsa T, Maymon E, et al. Funisitis and chorionic vasculitis: the histological counterpart of the fetal inflammatory response syndrome. J Matern Fetal Neonatal Med 2002;11:18–25.
7. Sampson JE, Theve RP, Blatman RN, et al. Fetal origin of amniotic fluid polymorphonuclear leukocytes. Am J Obstet Gynecol 1997; 176:77–81.
8. Romero R, Mazor M. Infection and preterm labor. Clin Obstet Gynecol 1988;3115:584.
9. Bashiri A, Burstein E, Mazor M. Cerebral palsy and fetal inflammatory response syndrome: a review. J Perinat Med 2006; 34:5–12.
10. Liu Z, Tang Z, Li J, Yang Y. Effects of placental inflammation on neonatal outcome in preterm infants. Pediatr Neonatol 2014;55: 35–40.
11. Park CW, Lee SM, Park JS, et al. The antenatal identification of funisitis with a rapid MMP-8 bedside test. J Perinat Med 2008;36: 497–502.
12. Park CW, Yoon BH, Park JS, Jun JK. A fetal and an intra-amniotic inflammatory response is more severe in preterm labor than in preterm PROM in the context of funisitis: unexpected observation in human gestations. PLoS One 2013;8:e62521.
13. Rovira N, Alarcon A, Iriondo M, et al. Impact of histological chorioamnionitis, funisitis and clinical chorioamnionitis on neurodevelopmental outcome of preterm infants. Early Hum Dev 2011; 87:253–7.
14. Yoon BH, Romero R, Park JS, et al. Fetal exposure to an intra-amniotic inflammation and the development of cerebral palsy at the age of three years. Am J Obstet Gynecol 2000;182:675–81.
15. Yoon BH, Oh SY, Romero R, et al. An elevated amniotic fluid matrix metalloproteinase-8 level at the time of mid-trimester genetic amniocentesis is a risk factor for spontaneous preterm delivery. Am J Obstet Gynecol 2001;185:1162–7.
16. Yoon BH, Romero R, Kim CJ, et al. Amniotic fluid interleukin-6: a sensitive test for antenatal diagnosis of acute inflammatory lesions of preterm placenta and prediction of perinatal morbidity. Am J Obstet Gynecol 1995;172:960–70.
17. Coultrip LL, Lien JM, Gomez R, et al. The value of amniotic fluid interleukin-6 determination in patients with preterm labor and intact membranes in the detection of microbial invasion of the amniotic cavity. Am J Obstet Gynecol 1994;171:901–11.
18. Oh KJ, Park KH, Kim SN, et al. Predictive value of intra-amniotic and serum markers for inflammatory lesions of preterm placenta. Placenta 2011;32:732–6.
19. Minagawa K, Tsuji Y, Ueda H, et al. Possible correlation between high levels of IL-18 in the cord blood of pre-term infants and neonatal development of periventricular leukomalacia and cerebral palsy. Cytokine 2002;17:164–70.
20. Romero Gómez MP, García-Perea A, Ruiz Carrascoso G, et al. Campylobacter fetus peritonitis and bacteremia in a patient undergoing continuous ambulatory peritoneal dialysis. J Clin Microbiol 2010;48:336–7.
21. Ville Y. [Premature delivery and inflammation]. J Gynecol Obstet Biol Reprod (Paris) 2001;30:12–16.
22. Lee J, Kim JS, Park JW, et al. Chronic chorioamnionitis is the most common placental lesion in late preterm birth. Placenta 2013;34: 681–9.
23. Brown AS, Hooton J, Schaefer CA, et al. Elevated maternal interleukin-8 levels and risk of schizophrenia in adult offspring. Am J Psychiatry 2004;161:889–95.
24. Gomez R, Romero R, Ghezzi F, et al. The fetal inflammatory response syndrome. Am J Obstet Gynecol 1998;179:194–202.
25. Kacerovsky M, Cobo T, Andrys C, et al. The fetal inflammatory response in subgroups of women with preterm prelabor rupture of the membranes. J Matern Fetal Neonatal Med 2013;26:795–801.
26. Satar M, Turhan E, Yapicioglu H, et al. Cord blood cytokine levels in neonates born to mothers with prolonged premature rupture of membranes and its relationship with morbidity and mortality. Eur Cytokine Netw 2008;19:37–41.
27. Cift T, Uludag S, Aydin Y, Benian A. Effects of amniotic and maternal CD-146, TGF-b1, IL-12, IL-18 and IFN-g, on adverse pregnancy outcome. J Matern Fetal Neonatal Med 2013;26:21–5.
28. Ekelund CK, Vogel I, Skogstrand K, et al. Interleukin-18 and interleukin-12 in maternal serum and spontaneous preterm delivery. J Reprod Immunol 2008;77:179–85.
29. Edwards RK, Clark P, Locksmith Gregory J, Duff P. Performance characteristics of putative tests for subclinical chorioamnionitis. Infect Dis Obstet Gynecol 2001;9:209–14.

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LC−MS-Based Metabolomics Identification of Novel Biomarkers of Chorioamnionitis and Its Associated Perinatal Neurological Damage

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*Supporting Information

ABSTRACT: Chorioamnionitis is a complication of pregnancy associated with significant maternal and perinatal long-term adverse outcomes. We apply high-throughput amniotic fluid (AF) metabolomics analysis for better understanding the pathophysiological mechanism of chorioamnionitis and its associated perinatal neurological injury and to provide meaningful information about new potential biomarkers. AF samples (n = 40) were collected from women at risk of chorioamnionits. Detailed clinical information on each pregnancy was obtained from obstetrical and neonatal medical examination. Liquid chromatography (LC)/mass spectrometry (MS) followed by data alignment and filtration as well as univariate and multivariate statistical analysis was performed. Statistically significant differences were found in 60 masses in positive and 115 in negative ionization mode obtained with LC/quadrupole time-of-flight MS (LC−QTOF-MS) between women with and without chorioamnionitis. Identified compounds were mainly related to glycerophospholipids and sphingolipids metabolism. From them, LPE(16:0)/LPE(P-16:0) and especially lactosylceramides emerged as the best biomarker candidates. Sulfocholic acid, trioxocholenoic acids, and LPC(18:2) were particularly increased in women with chorioamnionitis whose newborns developed perinatal brain damage. Therefore, we propose LPE(16:0)/LPE(P-16:0) and lactosylceramides as biomarkers for chorioamnionitis as well as LPC(18:2), trioxocholenoic acid, and sulfocholic acid for its associated perinatal brain damage. Metabolomics fingerprinting of AF enables the prediction of pregnancy-related disorders and the development of new diagnostics strategies.

KEYWORDS: *metabolomics, metabolic fingerprinting, chorioamnionitis, amniotic fluid, perinatal neurological damage, perinatology, biomarkers*

INTRODUCTION

Perinatal brain injury remains one of the main causes of life-long neurological disability. Although its etiology is multifactorial, prematurity and chorioamnionitis are well-known risk factors for the appearance of this condition. In fact, perinatal infection appears to play a key role in the pathogenesis of perinatal neurological injury in preterm infants. The development of brain damage in these babies may be mediated by a fetal inflammatory response, as evidenced by increased concentration of cytokines in the amniotic fluid (AF) or umbilical cord blood.[1,2] In addition to the preterm infant exposure to proinflammatory factors, the high frequency of brain injury in these children may be due to insufficient levels of developmentally regulated protective substances such as thyroid hormones or glucocorticosteroids. It is an extended idea that some biomolecules may either promote or protect against brain damage in the preterm neonate. Investigation along this line could help to find new biomarkers to predict and to better understand the pathophysiology of these conditions. This could help to prevent the risk of cerebral palsy in very preterm babies.[3]

The current tests with the best sensitivity (Se) and Specificity (Sp) for diagnosing subclinical chorioamnionitis on AF include: Gram stain (Se 24%, Sp 99%), leukocyte count (= >30/mm^3) (Se 57%, Sp 74%), glucose concentration (<10 mg/dL)(Se 57%, Sp 78%), and if available IL-6 concentration (>7.9 ng/mL) (Se 81%, Sp 75%).[4] The best performance is given by IL-6, but it is not available in all laboratories, the results have been very variable according to different studies, the cutoff level is not clear, and specificity may be very low in some cases as elevations of proinflammatory cytokines may be present in many other situations such as obesity or the metabolic syndrome.[5] To our knowledge, there is not a clinical test to predict neurological injury performed in either maternal samples or in AF. It is known that proinflammatory cytokines may be elevated in AF in cases of perinatal injury associated with chorioamnionitis, but its application still remains in the experimental setting. Currently, there is no possibility to predict these anomalies. Images technology such as 2D and 3D

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Table 1. Clinical Characteristics and Results According to the Presence of Chorioamnionitis and Perinatal Neurological Injury[a]

parameter	Excluded $n = 12$	Control $n = 13$	Chorio Not PNI $n = 9$	Chorio + PNI $n = 6$	intergroups differences, P value
maternal age (years)	32.08 ± 5.74	31.31 ± 5.76	32.56 ± 4.27	32.67 ± 6.61	0.27
nulliparity (n, %)	8, 66.7%	8, 61.5%	7, 77.8%	4, 66.7%	0.58
gestational age at amniocentesis (weeks)	27.05 ± 3.34	27.75 ± 2.49	26.38 ± 2.05	25.60 ± 1.32	0.35
preterm labor (n, %)	12, 100%	5, 38.5%	9, 100%	6, 100%	<.0001
PPROM (n, %)	12, 100%	7, 53.8%	6, 66.7%	4, 66.7%	0.07
gestational age at delivery (weeks)[b]	29.59 ± 1.99	35.46 ± 3.41	28.43 ± 2.54	26.33 ± 1.53	<.0001
birthweight (g)[c]	1870 ± 639	2702 ± 771	1171 ± 447	774 ± 71	<.0001
AF glucose (mg/dL)[d]	22.25 ± 14.37	32.33 ± 14.55	15.50 ± 16.52	11.89 ± 9.49	0.01
AF leukocytes (cells/mL)[e]	10 (5−71.25)	5 (0−11.50)	30 (9−870)	221.5 (113.25−725)	0.002
AF IL-6 (pg/mL)[f]	198.50 (67.79−392.25)	48.96 (29.97−69.86)	866 (414.50−1105.50)	1048.50 (809.75−1686.25)	<.0001

[a]Post hoc analysis. [b]Gestational age at delivery: Excluded vs Control, $P < 0.0001$; Excluded vs Chorio + PNI, $P = 0.089$; Excluded vs Not PNI Chorio, $P = 0.99$; Control vs Chorio + PNI, $P < 0.0001$; Control vs Not PNI Chorio, $P < 0.0001$; Chorio + PNI vs Not PNI Chorio, $P = 0.75$. [c]Birth weight: Excluded vs Control, $P = 0.30$; Excluded vs Chorio + PNI, $P = 0.14$; Excluded vs Not PNI Chorio, $P = 0.60$; Control vs Chorio + PNI, $P < 0.0001$; Control vs Not PNI Chorio, $P = 0.001$; Chorio + PNI vs Not PNI Chorio, $P = 0.99$. [d]Glucose: Excluded vs Control, $P = 0.49$; Excluded vs Chorio + PNI, $P = 0.99$; Excluded vs Not PNI Chorio, $P = 0.58$; Control vs Chorio + PNI, $P = 0.12$; Control vs Not PNI Chorio, $P = 0.01$; Chorio + PNI vs Not PNI Chorio, $P = 0.99$. [e]Leukocytes: Excluded vs Control, $P = 0.10$; Excluded vs Chorio + PNI, $P = 0.01$; Excluded vs Not PNI Chorio, $P = 0.14$; Control vs Chorio + PNI, $P < 0.0001$; Control vs Not PNI Chorio, $P = 0.01$; Chorio + PNI vs Not PNI Chorio, $P = 0.32$. [f]IL-6: Excluded vs Control, $P = 0.99$; Excluded vs Chorio + PNI, $P < 0.0001$; Excluded vs Not PNI Chorio, $P = 0.004$; Control vs Chorio + PNI, $P < 0.0001$; Control vs Not PNI Chorio, $P < 0.0001$; Chorio + PNI vs Not PNI Chorio, $P = 0.23$.

ultrasound and MRI scan can detect very early stages of these conditions, but this is when they are already present, which represents an early diagnosis rather than a prediction. There have been some attempts to evaluate the potential usefulness of several brain proteins such as S100B, enolase, and glial fibrillary protein but specifically in hypoxic insult of the brain rather than focusing in the inflammatory origin.[4,5]

Metabolomics and proteomics are two promising young technologies that provide a specific phenotypic fingerprinting for complex diseases such as perinatal brain injury. Recent studies have validated the enormous potential waiting to be revealed for these technologies.[6] In the obstetrics field, both proteomics and metabolomics could reveal complex interactions between mother, placenta, and fetus, which allow us to identify key features for prediction, "real-time" monitoring, and diagnosis of chorioamnionitis and associated comorbidities such as brain damage. In the present study, we explored the potential of metabolomics of AF to identify women with histological chorioamnionitis and associated perinatal brain injury.

MATERIAL AND METHODS

Study Population, Sample Collection, and Histological Study of the Placenta

Forty pregnant women at high risk of preterm labor and chorioamnionitis were enrolled for the study. Inclusion criteria included: preterm premature rupture of membranes (gestational age between 24 and 32 weeks), and/or preterm labor with poor prognosis (gestational age between 24 and 28 weeks, refractory to tocolysis, prolapsed amniotic sac in vagina, analytical suspected infection, and/or metrorrhagia of unknown cause). Amniocentesis procedures were clinically indicated and carried out for evaluating AF infection. An extra amount of 3 mL of AF was obtained after written informed consent. After amniocentesis, AF was immediately transported in a capped sterile syringe to the biobank, and all samples were stored at −80 °C until analysis. The study was approved by the Local Ethical and Research Committees, and all women signed a written consent form for participating. All samples were collected in the Division of Maternal and Fetal Medicine, University Hospital La Paz in Madrid.

Clinical characteristics of the study participants are presented in Table 1. Clear classification of cases is extremely important in metabolomics analysis for a proper evaluation of the differences in metabolites between groups and to construct the predictive models. For that, not only histological analysis, but also clinical characteristics were taken into account.

According to clinical data and obstetrical characteristics of the studied population, the samples were divided as follows: control samples, C ($n = 13$), which were obtained from women who tested negative for both microbiological and histological studies, had babies with normal neurological evaluation and absence of neonatal signs of infection; CHT (total number of chorioamnionitis samples positive for histological methods) ($n = 15$). The CHT group was subdivided into two groups according to the presence or absence of perinatal brain injury (CHN vs CH). In total, 12 samples negative for histological chorioamnionitis were excluded for the comparative analysis (not possible to consider them as adequate controls). The samples were excluded based on the following criteria: six cases of positive AF PCR (all of them to *Ureaplasma urealyticum*), six cases of mild periventricular ecodensities, one case of periventricular leukomalacia, two cases of IVH grade I−II, and two cases of IVI grade III−IV.

Perinatal brain injury was defined as the presence of either intraventricular hemorrhage or periventricular leukomalacia on the bases of postnatal ultrasound or MRI scans.

Pregnancies were followed up and after delivery, detailed histologic examinations of placentas/umbilical cords were performed. Histological study included three sections of umbilical cord (proximal to the placenta, middle, and proximal to fetus), one membrane roll (by "jelly roll" method in order to

obtain a maximum amount of membranes with decidua capsularis), and three full-thickness sections of parenchyma, including chorionic plate vessels.

Microbiological Studies

Molecular analysis: totalDNAwas extracted from 400 mL of AF samples and eluted in 100 mL using a MagNA Pure Compact system and the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, Diagnostics GmbH, Mannheim, Germany). Detection of bacterial DNA was done by broad-range real-time PCR targeting the 16S rRNA gene using Takara Premix Ex Taq and oligonucleotides P891F, P1033R, and the Taqman probe Uniprobe.[7] All the positive samples were identified by amplification and pyrosequencing of three short regions of the 16S rRNA gene as described.[8]

AF was cultured for aerobic and anaerobic bacteria, and Gram staining was performed for direct examination of Gram +. The culture medium used for the detection of urogenital mycoplasmas is a commercial medium "Mycofast Evolution 2" (ELITech France SAS).

In addition, AF glucose concentration and leukocyte count were determined by using standard automatized methods. Concentrations of IL-6 were determined by ELISA.

Metabolic Fingerprinting of Amniotic Fluid with LC–QTOF-MS

Sample Preparation. Sample preparation was done according to LC–MS standard protocol in our research center CEMBIO.[9] Briefly, for LC–MS analysis, proteins were precipitated by mixing one volume of plasma with three volumes of cold (−20 °C) mixture of methanol/ethanol (1:1). Samples were vortex-mixed and stored at −20 °C for 5 min. After centrifugation at 16 000*g* for 10 min at 4 °C (centrifuge 5415 R Eppendorf), the supernatant was filtered using a 0.22 μm nylon filter.

QC samples were prepared by pooling equal volumes of each sample and were injected at the beginning and the end of each analysis and every five sample injections to provide a measurement of the system's stability and performance as well as reproducibility of the sample treatment procedure.[10]

Chemicals and Reagents. Ultrapure deionized water obtained by Milli-Q plus 185 system (Millipore, Billerica, MA, USA) was used for preparation of all the buffers. Standards, such as histidine, creatine, and bilirubin, and LC–MS grade organic solvents, ethanol, acetonitrile, methanol, and formic acid were purchased from Fluka Analytical (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The reference mass solution kit for LC–MS containing purine, hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (HP-921), and ammonium trifluoroacetate ($TFANH_4$) was obtained from Agilent Technologies (USA).

LC–QTOF-MS Analysis. The UHPLC system (Agilent 1290 Infinity LC System) consisted of a degasser, two binary pumps, and a thermostated autosampler (maintained at 4 °C) coupled with LC–QTOF-MS (6550 iFunnel) system (Agilent Technologies) was used for AF fingerprinting. A 0.5 μL sample of extracted AF samples was injected to a thermostated at 60 °C reverse-phase Zorbax Extend C_{18} column (2.1 mm × 50 mm, 1.8 μm; Agilent Technologies). The flow rate was 0.6 mL/min with solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The chromatographic gradient started at 5% phase B up to the first minute, increased to 80% from 1–7 min, then to 100% from 7–11.5 min, and returned to 5% of phase B from 12–15 min (system re-equilibration). The system was operated in full scan mode from 50–1000 m/z for positive and 50–1100 m/z for negative mode. Capillary voltage was set to 3 kV for positive and negative ionization mode; the drying gas flow rate was 12 L/min at 250 °C and gas nebulizer 52 psi, fragmentor voltage 175 V for positive and 250 V for negative ionization mode, skimmer 65 V and octopole radio frequency voltage (OCT RF Vpp) 750 V. Data were collected in the centroid mode at a scan rate of *1.0* spectrum/s. Accurate mass measurements were obtained by means of an automated calibrant delivery system using a Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI) source that continuously introduces a calibrant solution that contains reference masses at m/z 121.0509 ($C_5H_4N_4$) and m/z 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for positive and m/z 112.9856 8 ($C_2O_2F_3(NH_4)$) and 1033.9881 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for negative ionization mode. Samples were analyzed in a separate runs (positive and negative ion mode) in a randomized order.

Data Acquisition and Statistical Analysis. Raw data acquired using UHPLC–MS system were processedto provide structured data in an appropriate format for data analysis. The data collected by LC–MS were cleaned of background noises and unrelated ions by the molecular feature extraction (MFE) tool in the MassHunter Qualitative Analysis Software (B.05.00, Agilent Technologies). The MFE algorithm uses the accuracy of the mass measurements to group ions related by their charge-state envelope, isotopic distribution, and the presence of adducts and dimmers and in that way the list of all possible components, as represented by the full TOF mass spectral data, was created. Each compound is described by mass, retention time, and abundance.

Alignment of drift (by retention time and mass) and data filtering were performed with Mass Profiler Professional B.12.1 (Agilent Technologies) software. Before statistical analysis, filtration of data matrix by samples frequency was also applied. For each comparison (C vs CHT, C vs CH, C vs CHN, CH vs CHN), only those variables that were present in 100% of the samples in at least one of the group were selected and considered for furtheranalysis.

For univariate statistical analysis, data normality was verified by evaluation of the Kolomgorow–Smirnov–Lillefors and Shapiro–Wilk tests and variance ratio by the Levene's test. Numerical data are shown as mean ± SD or as median and interquartile range according the variable distribution. Differences between experimental groups were evaluated by unpaired *t* test (equal or unequal variance) or nonparametric (Mann–Whitney test) with post hoc Benajmini Hochberg (FDR) and Bonferroni test. When more than two groups were compared, intergroups differences were tested by using either the ANOVA or the Kruskal–Wallis tests according to the distribution, normal or not, of the variables. Post hoc analysis was done as mentioned before. The levels of statistical significance were set at 95% level ($P < 0.05$). Statistical analyses were performed using Matlab R2010a (Mathworks) software. MetaboAnalyst and ROCCET data annotation approach was used for testing the relationships between variables by simple Spearman partial correlation coefficient and to perform receiver operating characteristic ROC analysis.[11,12] The optimized cutoff values in this study were those corresponding with the highest accuracy (maximum sensitivity and specificity). Multivariate (unsupervised and supervised) analysis as well as other multivariate calculation and plots was performed by using SIMCA-P+ 12.0 (Umetrics, Umea, Sweden).

Compound Identification. Identification of compounds that were found to be significant in class separation was performed by searching accurate masses against the online available databases as METLIN (http://metlin.scripps.edu), KEGG (http://www.genome.jp/kegg/genome.html),and LIPIDMAPS (http://www.lipidmaps.org/) by the in-house developed CEU mass mediator (http://ceumass.eps.uspceu.es/mediator). Information from HMDB (http://hmdb.ca) was also considered for compound identification. The identity of compounds was confirmed by LC−MS/MS by using a QTOF (6550 system, Agilent Technologies) with the same chromatographic conditions as the primary analysis. Ions were targeted by collision-induced dissociation (CID) fragmentation on the fly based on the previously determined accurate mass and retention time. Accurate mass and isotopic distributions for the precursor and product ion have been studied for final confirmation of the particular compounds. Confirmation with standards was performed by comparison of retention time, isotopic distribution, and fragments of commercially available reagents (bilirubin, histidine, creatine) with those obtained in analyzed samples.

Experiment Validation. PLS-DA models that were obtained according to multivariate calculations were validated by cross-validation tool.[13] Validation was performed by using the leaving-1/3-out approach. A randomized data set was divided into three parts, and 1/3 of samples were excluded to build a model with the remaining 2/3 of samples. Then, the excluded samples were predicted by the new model, and the process was repeated until all samples have been predicted at least once. Each time the percentage of correctly classified samples was calculated.

RESULTS

Clinical Results

There were 15 cases of histological chorioamnionitis (37.5%). PCR was positive in 18 cases (45%), 14 of them to *Ureaplasma urealyticum*, one to *Lactobacillus*, one to *Propinibacterium acnes*, one to *Streptococcus anginosus*, and one to *Cytomegalovirus*. Cultures were positive in only six cases (two cases to *Ureaplasma urealyticum*, one to *Mycoplasma hominis*, one to *Lactobacillus jensenii*, one to *Prevotella*, and one to *Streptococcus anginosus*).

Gestational age at delivery was 30.4 ± 4.1 weeks. This was significantly lower in cases of chorioamnionitis (27.6 ± 2.4 vs 32.3 ± 3.9; $P < 0.0001$). Preterm delivery rate was 100% in the chorioamnionitis group and 72% (18/25) in the non-chorioamnionitis group ($P = 0.03$). Birth weight was 1698.4 ± 870.6 g, and it was also significantly lower in cases of chorioamnionitis (1069.9 ± 423.2 vs 2169.7 ± 823.8; $P < 0.0001$).

In total, there were 16 cases of totally normal neurological evaluation (40%). In the other 24 cases, there were some neurological findings including: four cases (10%) of IVI grade I−II, four cases of IVI grade III−IV, 14 cases of mild periventricular echodensities, and two cases of periventricular leukomalacia. There were statistically significant differences in the perinatal brain evaluation between cases with or without chorioamnionitis ($P = 0.001$) (Table 2). In total, there were six cases of perinatal neurological injury in the group of chorioamnionitis (40%) in comparison with four cases in the group without chorioamnionitis (16%).

Table 2. Result of Perinatal Neurological Evaluation[a]

parameter	nonchorioamnionitis	chorioamnionitis	total cases
normal	15 (60%)	1 (6.7%)	16
IVI grade I−II	1 (4%)	3 (20%)	4
IVI grade III−IV	2 (8%)	2 (13.3%)	4
periventricular leukomalacia	1 (4%)	1 (6.7%)	2
periventricular echodensities	6 (24%)	8 (53.3%)	14
total	25 (100%)	15 (100%)	40

[a]$P = 0.001$, statistically significant differences in the perinatal brain evaluation between cases with or without chorioamnionitis.

Clinical characteristics and results of the AF analysis according to the presence or not of both chorioamnionitis with and without perinatal neurological injury as well as in cases that were excluded for the metabolomics comparative analysis are shown in Table 1. In comparison with controls, gestational age at delivery and birth weight were significantly decreased in both groups of chorioamnionitis (with or without PNI), AF glucose was significantly decreased in cases of chorioamnionitis without PNI, and both leukocytes and IL-6 were increased in both groups of chorioamnionitis. None of the clinical variables were significantly different between women with chorioamnionitis with or without PNI.

Other perinatal outcomes included six cases (15%) of necrotizing enterocolitis, three cases of neonatal sepsis (7.5%), and 10 cases (25%) of late nosocomial sepsis.

Metabolomics Results

UHPLC−QTOF-MS system operated both in positive and in negative scan mode has been applied for AF fingerprinting. In total, 40 samples were analyzed; however, because of the examination of clinical data, 12 samples were excluded from the data set. For the samples considered for data treatment ($n = 28$), cross-validation tools as described in Materials and Methods were used to estimate the predictive ability of the PLS-DA models based on the filtered data matrix. For data recorded in LC−MS ESI(+) mode, samples were classified correctly in 80 ± 9%, and for ESI(−) in 85 ± 10%.

Unsupervised principal component analysis (PCA-X) models were used for plotting QC samples. Very good clustering was observed in both ESI(+) and ESI(−) modes that reflects the system's stability, performance, and reproducibility of the sample treatment procedure (Figure 1).[10] Variation of the compound concentrations in QC samples expressed as relative standard deviation (%RSD) was also calculated. A threshold of 30% was set for the RSD values of metabolites in the QC samples.

To perform samples classification, the data set was filtered by choosing only metabolic features present in 100% of the samples in at least one of the groups in specified interpretations. For those data sets, 827 (C vs CHT), 994 (C vs CH), 1055 (C vs CHN), and 1162 (CH vs CHN) entities were obtained in ESI(+), and 407 (C vs CHT), 471 (C vs CH), 597 (C vs CHN), and 638 (CH vs CHN) entities were found in ESI(−).

In unsupervised PCA-X analysis with Hotelling's T^2 Range test, four outliers were detected (one ESI(+) and three ESI(−)) that belonged to the samples discharged due to clinical characteristics. Additionally, supervised partial least squares discriminant analysis (PLS-DA) and orthogonal PLS (OPLS-

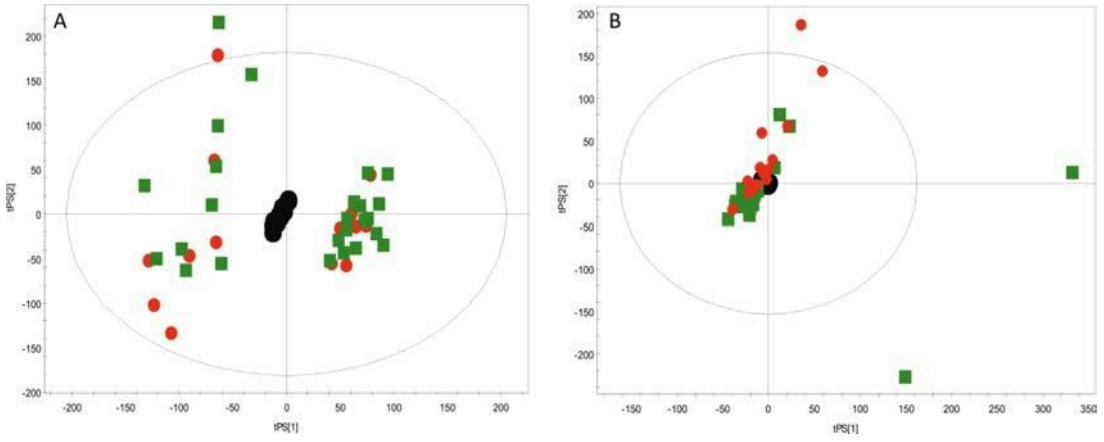

Figure 1. PCA-X score plots for all samples included to the study (green squares, control; red circles, chorioamnionitis; black circles, predicted QC samples). (A) LC−MS ESI(+) and (B) LC−MS ESI(−).

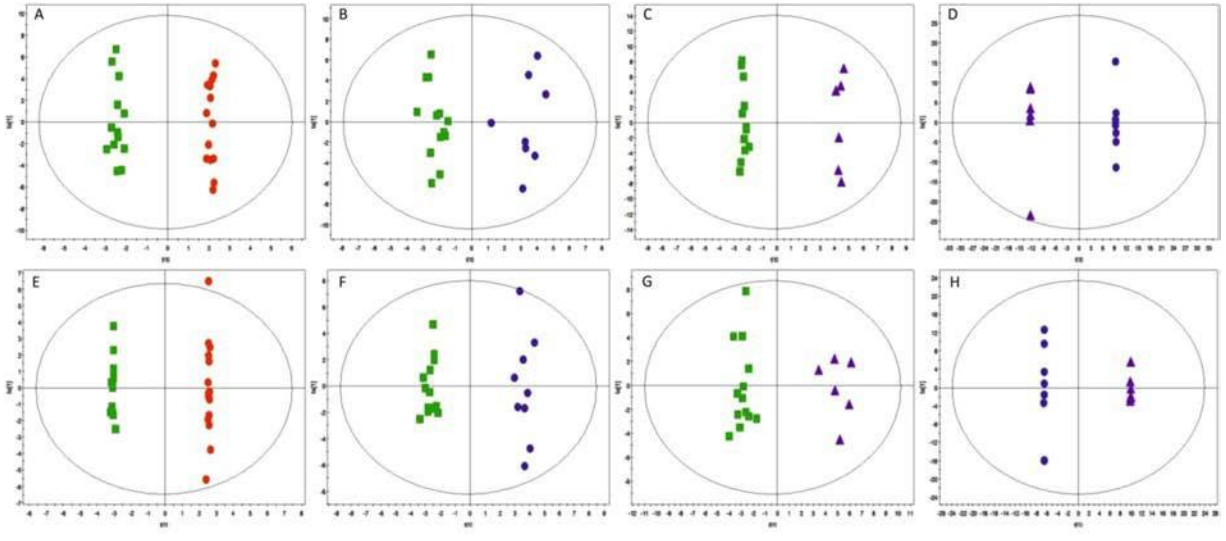

Figure 2. Supervised OPLS-DA multivariate analysis (green squares, control C; red circles, all chorioamnionitis samples CHT, black circles, chorioamnionitis CH; and purple triangles, chorioamnionitis + neurological complications CHN). Plots A−D represent the data obtained in ESI(+) in the following interpretation, C vs CHT with quality of variance explained and variance predicted (R^2 = 0.99, Q^2 = 0.64), C vs CH (R^2 = 0.93, Q^2 = 0.52), C vs CHN (R^2 = 0.99, Q^2 = 0.49), CH vs CHN (R^2 = 0.99, Q^2 = 0.7). Plots E−H represent the data obtained in ESI(−) in the following interpretation, C vs CHT with quality of variance explained and variance predicted (R^2 = 0.99, Q^2 = 0.76), C vs CH (R^2 = 0.98, Q^2 = 0.64), C vs CHN (R^2 = 0.95, Q^2 = 0.69), CH vs CHN (R^2 = 0.99, Q^2 = 0.8).

DA) discriminant analysis were used for modeling the differences between disease and control groups. All applied models show clear separation of samples. The parameters of obtained models were satisfied with good quality of variance explained (R^2) and variance predicted (Q^2) (Figure 2). Statistical analysis was performed as described in Materials and Methods yielding, respectively, 60 (C vs CHT), 49 (C vs CH), 68 (C vs CHN), and 22 (CH vs CHN) statistically significant features in LC−MS ESI(+), and 115 (C vs CHT), 66 (C vs CH), 94 (C vs CHN), and 18 (CH vs CHN) statistically significant features in LC−MS ESI(−). Identification of those metabolites was performed as described in the Materials and Methods section. For all identified metabolites, calculated mass error of measured mass in comparison to monoisotopic mass was calculated. Identified metabolites are summarized in Table 3, including retention time, the monoisotopic mass, percentage of changes between groups, and statistical significance. In total, 32 compounds have been identified in AF to be statistically different between the groups as respond to choriamnionitis (Figure 3). The majority (41%) of detected metabolites belong to choline containing compounds, including lysophosphatidylcholines (16%), lysophosphatidylethanolamines (19%), lysophosphatidylserine (3%), and phosphatidylcholine (3%). Sphingolipids (sphingomyelins and ceramides) constitute 13%. Other compounds such as those involved in bile acids, vitamin D3, or pyruvate metabolism and other metabolic pathways were also found (Figures 3 and 4). The most pronounced choriamnionitis specific changes corresponded to lysophosphatidylcholines, lysophosphatidylethanolamines (Figure 5), and lactosylceramides. Lysophosphatidylcholines with saturated 16:0, 18:0, and unsaturated 18:1, 18:2, 20:4 fatty acid chains were followed by lysophosphatidylethanolamines with the same fatty acid composition (Table 3, Figure 5). Interestingly, the fatty acids with the 16:0 and 18:1 chains are also components of identified lysophosphatidylserine, phosphatidylcholine, sphingomyelins, and lactosylceramides. All lipids related compounds, especially lactosylceramides, exhibited the highest significant differences

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Table 3. List of Statistically Significant Compounds Identified by a LC−MS with Regulation in the Specified Interpretations

compound category	identification[e]	monoisotopic mass (Da)	formula	RT	mass error (ppm)	RSD in QC	FC and *P* value[a] C vs CHT	C vs CH	C vs CHN	CH vs CHN
Glycerophospholipids										
	LPC(16:0)	495.3325	C24H50NO7P	5.6	0	11.3	+4.3 (0.00296)[b]	+5.0 (0.00084)	+3.3 (0.00766)	−1.5 NS
	LPC(18:0)	523.3638	C26H54NO7P	6.3	−5	10.3	+3.3 (0.00155)[b]	+3.0 (0.00799)	+3.7 (0.00194)	+1.2 NS
	LPC(18:1)	521.3481	C26H52NO7P	5.8	1	18.3	+4.1 (0.00033)[b]	+4.3 (0.00399)	+3.7 (0.00014)[b]	−1.2 NS
	LPC(18:2)	519.3325	C26H50NO7P	5.4	2	20.3	+4.1 (0.00028)[c]	+3.4 (0.00124)	+5.1 (0.00011)[c]	+1.5 NS
	LPC(20:4)	543.3325	C28H50NO7P	5.4	−3	13.6	+2.8 (0.00118)	+3.1 (0.00790)	+2.5 (0.00160)	−1.2 NS
	LPE(16:0)	453.2855	C21H44NO7P	5.6	−4	9.6	+3.1 (0.00522)[b]	+3.2 (0.01898)	+2.9 (0.00862)	−1.1 NS
	LPE(P-16:0)	437.2906	C21H44NO6P	5.8	1	7.2	+5.1 (0.00023)[b]	+6.4 (0.00400)	+3.1 (0.02302)	−2.1 NS
	LPE(18:0)	481.3168	C23H48NO7P	6.2	2	14.2	+4.9 (0.00086)[b]	+5.0 (0.00618)	+4.5 (0.00033)[b]	−1.1 NS
	LPE(18:1)	479.3012	C23H46NO7P	5.8	1	10.8	+5.3 (0.01471)[b]	+6.4 (0.05455)	+3.8 (0.02381)	−1.7 NS
	LPE(O-18:1)/LPE(P-18:0)	465.3219	C23H48NO6P	6.4	1	8.8	+5.8 (0.00023)[b]	+7.6 (0.00237)	+3.2 (0.00014)[b]	−2.3 NS
	LPE(20:4)	501.2855	C25H44NO7P	5.3	1	12.7	+3.3 (0.00034)[b]	+4.3 (0.00494)	+1.7 (0.00005)[c]	−2.5 NS
	LPS(18:1)	523.2910	C24H46NO9P	6.8	6	15.4	+2.1 (0.00023)[b]	+2.5 (NS)	+1.6 (0.04717)	−1.6 NS
	PC(34:1)	759.5778	C42H82NO8P	11.9	−1	22.9	+12.0 (0.00056)[b]	+12.0 (0.04262)	+12.1 (0.00344)	+1.0 NS
Sphingolipids										
	SM(32:0)	674.5363	C37H75N2O6P	8.1	−5	5.7	+2.7 (0.00033)[b]	+2.8 (0.00616)	+2.5 (0.00081)	−1.1 NS
	SM(34:1)	702.5676	C39H79N2O6P	9.0	3	8.6	+3.4 (0.00082)	+3.9 (0.00883)	+2.7 (0.00176)	−1.5 NS
	LacCer(d18:1/16:0)[d]	861.6177	C46H87NO13	8.9	1	6.9	+35.1 (0.00002)[c]	+50.1 (0.00024)	+12.7 (0.00029)[b]	−3.9 NS
	LacCer(d18:1/24:1)[d]	971.7273	C54H101NO13	11.3	3	30.3	+16.4 (0.00003)[c]	+21.7 (0.00031)	+6.8 (0.00047)[b]	−3.2 NS
Sterol Lipids										
	deoxyvitamin D3	368.3443	C27H44	10.3	7	3.6	+2.8 (0.00014)	+3.1 (0.00272)	+2.5 (0.00006)	−1.3 NS
	dihydroxy-oxo-vitamin D3	430.3083	C27H42O4	5.5	−3	4.2	+2.1 (0.00207)	+1.8 (0.01463)	+2.5 (0.00140)	+1.4 NS
	dihydroxy-pregnane-glucuronide	496.3036	C27H44O8	4.1	−1	2.2	+1.2 (0.00047)[b]	+1.4 (NS)	+1.0 (NS)	−1.3 NS
	glycochenodeoxycholic acid Sulfate	529.2709	C26H43NO8S	4.2	1	5.6	+1.7 (0.01572)	+1.6 (NS)	+1.8 (NS)	+1.1 NS
	trioxo-cholenoic acid	400.2250	C24H32O5	4.8	9	10.1	+2.5 (0.00772)	+1.6 (NS)	+3.7 (0.00175)	+2.3 (0.013263)
	sulfocholic acid	488.2444	C24H40O8S	3.9	1	11.2	−1.3 NS	−1.6 (NS)	−1.0 NS	+1.6 (0.024268)
Tetrapyrroles and Derivatives										
	biliverdin IX	582.2478	C33H34N4O6	7.9	−4	9.2	+2.1 (0.00865)	+2.2 (NS)	+1.9 (0.00866)	−1.1 NS
	bilirubin (STD)	584.2635	C33H36N4O6	7.9	1	7.3	+2.1 (0.00003)[c]	+2.3 (0.00818)	+1.7 (0.02068)	−1.4 NS
Amino Acids and Derivatives										
	histidine (STD)	155.0695	C6H9N3O2	0.2	0	22.5	+1.5 (0.02327)	+1.6 (0.02419)	+1.4 (NS)	−1.2 NS
	creatine (STD)	131.0695	C4H9N3O2	0.3	0	29.0	+6.6 (NS)	+6.9 (NS)	+6.3 (0.02597)	−1.1 NS
Fatty Acyls										
	dimethyl arachidonoyl amine	331.2875	C22H37NO	7.1	−3	12.7	−1.2 (0.02363)	−1.1 (0.07085)	−1.3 (0.01498)	−1.1 NS
	hexacosanedioic acid	426.3709	C26H50O4	7.7	3	5.4	+1.8 (0.03810)	+1.6 (NS)	+2.1 (0.01347)	+1.3 NS
Others										
	hexose	180.0634	C6H12O6	0.2	2	14.1	−2.0 (0.01117)	−2.3 (0.00621)	−1.7 (NS)	+1.4 NS
	butynedioic acid	113.9953	C4H2O4	0.2	20	3.9	+1.1 (0.00327)[b]	+1.1 (0.03340)	+1.1 (NS)	−1.0 NS
	diacetylspermine	286.2369	C14H30N4O2	0.2	8	3.9	+1.9 (0.00087)	+2.2 (0.00341)	+1.6 (0.03495)	−1.4 NS

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Table 3. continued

[a]FC, fold change in the specified comparison, the sign indicates the direction of change in CHT, CH, or CHN group. [b]p values significant also due to FDR. [c]p values significant also due to Bonferroni test correction. [d]p values significant also due to possible different stereoisomeric form of glycosphingolipids. [e]LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; LacCer, lactosylceramide. STD, confirmed with standard.

in chorioamnionitis related cases as compared to control group (Table 3). In case of chorioamnionitis where neurological complications were present (CHN), we found LPC(18:0) and LPC(18:2) to be significantly altered (Table 3, Figure 5). The significant changes were also observed for trioxocholenoic and sulfocholic acids (Table 3).

Associations between identified compounds and glucose, leukocytes levels, and IL-6 concentrations were tested by Spearman correlation (Table S1, Supporting Information; Figure 6). All classes of lipids in CHT, CH, and CHN groups were negatively correlated with glucose level. The strongest correlations indicated in CH group were observed between glucose and LPE(P-16:0) (r_s = −0.8787), LacCer(d18:1/16:0) (r_s = −0.8285), LacCer(d18:1/24:1) (r_s = −0.8034), and glycochenodeoxycholic acid sulfate (r_s = −0.8452). LPE(P-16:0), LPE(20:4), and both LacCer compounds were highly correlated also with IL-6 and leukocytes level (Table S1, Supporting Information; Figure 6). Although highly elevated in chorioamnionitis, LPC(16:0) and LPC(18:1) do not exhibit highly significant correlations with IL-6, glucose, or leukocytes. In case of the CHN group, strong negative correlations with glucose were found for LPE(O-18:1)/LPE(P-18:0) (r_s = −0.9856), LacCer(d18:1/16:0) (r_s = −0.9276), deoxyvitamin D3 (r_s = −0.8987), PC(34:1) (r_s = −0.8971), LacCer(d18:1/24:1) (r_s = −0.8707), LPE(20:4) (r_s = −0.8117), and LPE(18:1) (r_s = −0.8117). Correlations with IL-6 were observed as negative association with lipid compounds with the highest values for LPC(18:2) (r_s = −0.7537), although LPC(18:2) was not correlated with glucose in CHN group (r_s = −0.3382). Worthy to consider is the fact that in the CHN group, there was no correlation observed for IL-6 and identified ceramides.

Additionally, we performed a ROC analysis with metabolites that showed the best correlations with CH and CHN conditions. The analysis revealed a high discriminant power for lysophosphatidylethanolamines (LPE(18:1), LPE(16:0), and LacCer(d18:1/16:0), LacCer(d18:1/24:1) with a AUC area >0.95 for CH outcomes. For the CHN group, LPC(18:2) exhibited a high discriminant power with a AUC area >0.99, which could explain the significant increase and significant correlations observed for that compound.

DISCUSSION

There are only a few reports studying metabolomics fingerprints in AF. Some of them have focused in the understanding of mechanisms leading to preterm delivery and in the prediction of this condition. In a recent review,[14] only six studies of metabolomics in AF were identified, and none of them were specifically designed for either women with chorioamnionitis or to look for markers and pathways of perinatal brain damage. The present study was conducted to evaluate metabolomics profiling of the AF to identify women with subclinical chorioamnionitis and those of them at the highest risks of this severecomplication.

The real clinical utility of the present study is first to help to identify new metabolic pathways and compounds that can be used as new biomarkers for subclinical chorioamnionits and also bring the opportunity to open new drug targeting. In this sense, the new metabolic pathways may be useful for better understanding not only chorioamnionitis, but also how intrauterine infection may cause complications such as very preterm premature rupture of membranes. Accumulation of some ceramides that can induce apoptotic processes on

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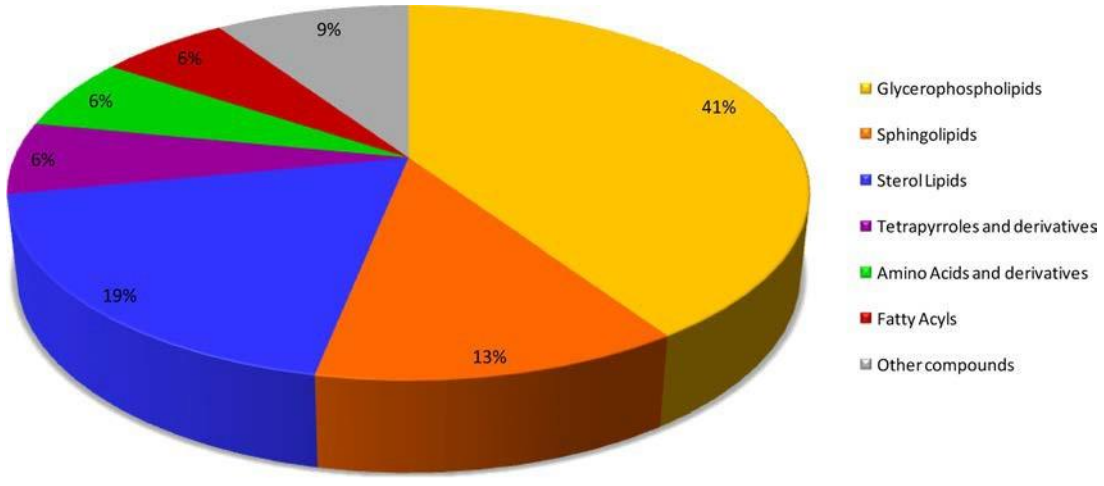

Figure 3. Distribution of all identified classes of metabolites found to be related to chorioamnionitis.

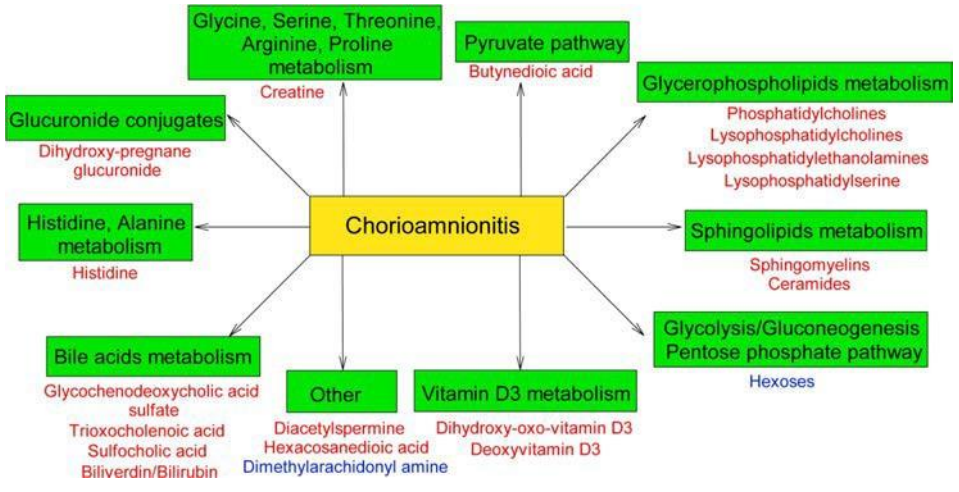

Figure 4. Schematic visualization of the major metabolic pathways and compounds altered by chorioamnionitis. Red-colored significant up-regulations and blue-colored down-regulations in the chorioamnionitis group compared with the controls.

amniotic membranes may be crucial to identify new drug targeting and to be used as biomarkers. Second, accumulation of some bile acids in a proinflammatory media may be also important for prediction and detecting newborns at special risks of intraventricular hemorrhage. This condition may be potentially prevented if a subgroup at special risk is identified.

Considering the metabolic components having significant differences between women with and without chorioamnionitis, we have identified several major metabolic pathways in chorioamnionitis. They have been summarized in Figure 4.

There are at least eight groups of metabolites that can be involved in the pathogenesis of this condition and also that can be potentially used as markers for this complication and its consequences. Some of the compounds are actually known to be altered in cases of chorioamnionitis. For example, the results showed that glucose levels (hexose group) are decreased in cases of chorioamnionitis, which is already known and used in the clinical practice. Nevertheless, the majority of the groups of metabolites are not so well identified as involved in the pathogenesis of the intrauterine infection.

One of the most important groups of metabolites showing strong significant differences between women with or without chorioamnionitis was the sphingolipids group, and more specifically within this metabolic pathways, sphingomyelin, and lactosylceramides. The latter showed the most important differences between both groups of all the analyzed metabolites, being the concentrations more than 3000 higher in the chorioamnionitis group. This led to the suggestion that lactosylceramides may be interesting molecules to be considered as biomarkers of this condition. Sphingolipids are a class of lipids derived from the aliphatic amino alcohol sphingosine. They are generally believed to protect the cell surface against harmful environmental factors by forming a mechanically stable and chemically resistant outer leaflet of plasma membrane. Sphingomyelin is one of the most abundant sphingolipids found in animals and is densely distributed in white matter of the brain where it plays a crucial role by insulating axons through formation of a membranous myelin sheat.[15] Under certain conditions, sphingomyelin is enzymatically degraded by the action of sphingomyelinase to ceramide, which is one of the best characterized bioactive sphingolipids that mediates intracellular signaling cascades. Ceramide can be further converted to various bioactive sphingolipids including lactosylceramides.[16,17]

Lactosylceramide plays an important role in inflammation and cell adhesion. It is considered as a proinflammatory mediator.[18] More specifically, TNF-alpha dose dependently stimulates the activity of lactosylceramide synthase and

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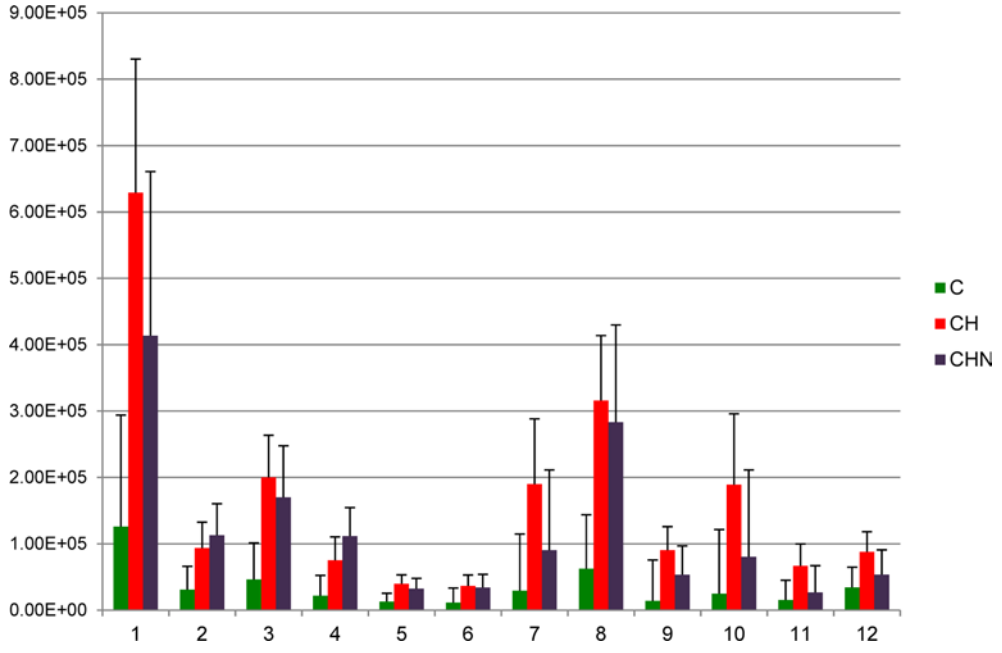

Figure 5. Comparison of average abundance of identified lysoglycerophospholipids: 1, LPC(16:0); 2, LPC(18:0); 3, LPC(18:1); 4, LPC(18:2); 5, LPC(20:4); 6, LPE(16:0); 7, LPE(P-16:0); 8, LPE(18:0); 9, LPE(18:1); 10, LPE(O-18:1)/LPE(P-18:0); 11, LPE(20:4); 12, LPS(18:1) in control (C), choriamnionitis (CH), and chorioamnionitis with neurological complication (CHN).

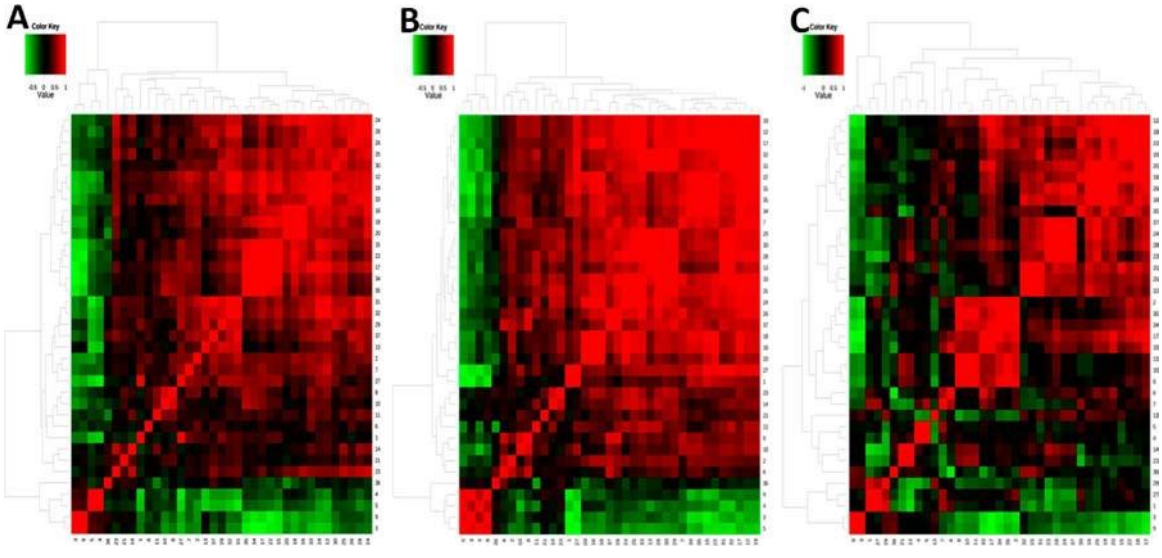

Figure 6. A color heatmap represents identified metabolites and glucose, leukocytosis, IL-6, newborn weight, and time of delivery Spearman's rank correlation analysis for the all CHT (A), CH (B), and CHN (C) samples. The colors refer to the pairwise correlation coefficient ranging from 1 (red) to −1 (green). (1, IL-6; 2, Leukocytosis; 3, Glucose; 4, Newborn weight; 5, Time of delivery; 6, Butynedioic acid; 7, Creatine; 8, Histidine; 9, Hexoses; 10, Diacetylspermine; 11, Dimethyl arachidonoyl amine; 12, Deoxyvitamin D3; 13, Hexacosanedioic acid; 14, Dihydroxy-oxo-vitamin D3; 15, LPE(P-16:0); 16, LPE(16:0); 17, LPE(O-18:1)/LPE(P-18:0); 18, LPE(18:1); 19, LPE(18:0); 20, LPC(16:0); 21, Dihydroxy-pregnane-glucuronide; 22, LPE(20:4); 23, LPC(18:2); 24, LPC(18:1); 25, LPS(18:1); 26, LPC(18:0); 27, Glycochenodeoxycholic Acid Sulfate; 28, LPC(20:4); 29, Biliverdin IX; 30, Bilirubin; 31, SM(32:0); 32, SM(34:1); 33, PC(34:1); 34, LacCer(d18:1/16:0); 35, LacCer(d18:1/24:1); 36, Trioxo-cholenoic acid; 37, Sulfocholic acid).

generates this ceramide. Then lactosylceramide activates superoxide production, nuclear factor kB, and ICAM-1 expression, which is accompanied by a significant increase in the adhesion of human neutrophils.[19] Lactosylceramide is a known physiological apoptotic agent that can increase prostaglandin release in parallel with induction of apoptosis in amnion epithelial cells, so amnios prostaglandin metabolism is linked with apoptosis in amnion epithelial cells and thus to membrane rupture. This action is mainly achieved in amnion epithelial cells rather than in mesenchymal cells.[20] Lactosylceramide was reported to be elevated in the AF of premature infants with rupture of membranes[21] and has been shown to activate NADPH oxidase-producing reactive oxygen species[22] known to induce apoptosis and stimulate cytokine production. In the present study, we found lactosylceramides to be highly increased in cases of chorioamnionitis and strongly correlated with IL-6 and glucose levels.

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It is remarkable that previously, Hallman et al. found that a high ceramide lactoside (greater than or equal to 5 nmol/mL) predicted chorioamnionitis with signs of infection at a sensitivity and a specificity of 94% and 95%, respectively, and a moderately high ceramide lactoside concentration (greater than or equal to 2.5 nmol/mL) predicted spontaneous preterm labor: sensitivity, 82%; specificity, 95%.[21] However, this excellent study was not really followed by clinical trials. Our results support these previous findings and indicate highly significant changes and strong correlation of LacCer(d18:1/16:0) and LacCer(d18:1/24:1) with already known diagnostics methods. We propose that lactosylceramides could be excellent biomarkers for predicting subclinical chorioamninitis. Interestingly, lactosylceramide has been considered also as a lipid second messenger in neuroinflammatory diseases.[23] It is an important signaling component for the induction of proinflammatory mediators and astrogliosis.[24] Cytokines produced under various disease conditions regulate the metabolism of sphingomyelin for generation of sphingolipids, and some of these metabolites participate in the up-regulation of the inflammatory process. More specifically, lactosylceramide promotes the expression of iNOS and production of nitric oxide induced by cytokines, particularly demonstrated in cultured astrocytes and in the brain. In the present study, we did not find differences in AF lactosylceramide between women with or without perinatal neurological injury in cases of chorioamnionits.[24] Even the level of the increase in cases of brain damage was lower, although not significantly lower than in cases without neurological harm. However, most of the cases defined as brain damage in our study were cases of IVH. While inflammation has been clearly related to the pathogenesis of other causes of neonatal brain damage such as periventricular leukomalacia and long-term sequels, there is not a clear relationship with the etiology of IVH. Therefore, we can conclude that lactosylceramide is highly increased in cases of chorioamnionitis, and it may be proposed as a candidate to be a biomarker of this condition; however, it seems to be not predictive for IVHcases.

Another group of metabolites showing significant differences between women with and without chorioamnionitis was related to glycerophospholipids, whose biological properties are mainly related to differences of the headgroup and fatty acids variations (different chain lengths, position, degrees of saturation, and double bond location).[25] Glycerophospholipids, including phosphatidylcholine (PC), lysophosphosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), and lysophosphatidylserine (LPS), are not only structural components of cellular membranes, but also serve as important as bioactive second messenger signaling molecules with wide-ranging biological effects.[25]

Phosphatidylcholine is the biosynthetic precursor of sphingomyelin (found to be up-regulated in chorioamnionitis) and has influence on the many metabolic pathways that constitute the sphingomyelin cycle. It is also a precursor for other glycerophospholipids like phosphatidic acid, lysophosphatidylcholine, and platelet-activating factor, each with important signaling functions, and of phosphatidylserine. Lysophosphosphatidylcholines (LPC) that were found significantly elevated in our study are membrane-derived signaling molecules generated from phospholipids through the enzymatic action of phospholipases A_2 (PLA_2) or phospholipases A_1 (PLA_1).[26] LPLs and related receptors have been found in a wide range of tissues and cells that indicate their importance in many physiological processes including inflammation, atherosclerosis, diabetes and obesity, cancer, or autoimmune diseases.[26−29] It is also demonstrated that subpopulation of human T-lymphocytes called natural killer (NKT) T-cells, with immunoregulatory properties, recognize lysophosphatidylcholine as an antigen presented by CD1d, which is an important route of NKT cells and cellular signaling pathways activation.[30,31] LPC has an established role as a proinflammatory molecule, joining thromboxanes, leukotriens, and prostaglandins and sharing common metabolic pathways and regulatory mechanisms.[32] LPC is generated in the brain under pathological conditions that are accompanied by elevated IL-1β. IL-1β, released from activated macrophages, contributes significantly to tissue damage in inflammatory, degenerative, and autoimmune diseases. Under pathological conditions, overstimulation of phospholipase A_2 results in breakdown of PC membrane and subsequent accumulation of LPC in the damaged tissue. Increased LPC concentrations were detected in the brain following ischemia, epilepsy, and inflammation.[33] It has been shown to promote demyelination in the nervous system.[32] Those findings correspond to our results, as we indicate highly significant up-regulation of LPC(18:2) and LPC(18:0) related to chorioamnionitis with neurological complications.

Alternations of the lysophosphatidylethanolamines (LPE), particulary LPE(P-16:0) and LPE(16:0), were the most prominent changes that highly correlated with IL-6, glucose, and lekocyte levels in chorioamnionitis group. LPE is formed by hydrolysis of phosphatidylethanolamine by the enzyme phospholipase A_2as part of a deacylation/reacylation cycle that controls its overall molecular species composition. The physiological significance of LPE still remains unknown. However, LPE(16:0) appears to be a marker metabolite that can be used to distinguish the different stages of hepatocarcinogenesis.[34] Lysophosphatidylserine (LPS), found to be elevated in chorioamnionitis, is the deacylated form of phosphatidylserine (PS). Hydrolysis of PS is performed by a secretory enzyme PS-specific phospholipase A_1, which is poorly expressed under normal conditions but dramatically upregulated by inflammatory stimuli.[26] LPS, correlated with inflammatory reactions, has been known as a signaling phospholipid in mast cell biology, markedly enhancing stimulated histamine release and eicosanoid production.[35] In fact, our results indicate significant increasing of histidine levels associated with chorioamnionitis. Menon et al. observed histidine metabolites to be associated with early spontaneous preterm birth;[36] however, in our study, we did not find a correlation with gestational age or newborn weight.

Metabolites coming from different metabolic pathways, such as butynedioic acid from the pyruvate pathway, creatine, vitamine D3 derivatives, glucuronide conjugates, and other compounds (Figure 4), were also found to be altered due to inflammatory process. Low hexoses concentration is explained by the fact that glucose, due to infection, is used as energy source by bacteria and neutrophils. In a previous study, we have reported that AF glucose levels are even lower in cases of fetal inflammatory response due to fetal infection demonstrated by funisitis.[37] Higher levels of compounds involved in bile acids metabolism have been also found in our study. The excretion of bile acids produced by the fetus is performed to a large extent by the placenta and the maternal liver; therefore, impairment in biliary excretion may lead to the accumulation of bile acids in the fetal liver and placenta. We do not know the reasons why

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bile acids accumulate in AF and presumably in the fetal compartment, but infection and or inflammation of the membranes and the placenta may lead to disturbances in the transfer and clearance of these bile acids from the fetal to the maternal compartment. Other explanations based on the metabolic role of bile acids, especially in energy metabolism, cannot be excluded. In cholestatic pregnant rats, hydrophobic bile acids accumulation induces impairment of the placental antioxidant system and causes oxidative damage. These alterations are accompanied by enhanced activation of the mitochondrial pathway of apoptosis.[38] Moreover, the accumulation of hydrophobic bile acids in the fetal compartment also causes marked oxidative damage and apoptosis in the fetal liver.[39] Bile acids accumulation may cause other complications in extrahepatic fetal tissues such as carciomyocytes inducing dysrhythmia or lung injury.[40] This bile acid toxicity has been related to not only membrane damage through their detergent action on lipid components, but also to nondetergent effects such as oxidative stress and apoptosis. In this sense, the role of hydrophilic bile acids such as ursodeoxycholic acid to prevent or treat the toxicity of hydrophobic bile acids should be evaluated.[41] In cases of chorioamnionitis, the only group of metabolites that was significantly different between women with or without fetuses who were going to develop a perinatal neurological injury was the group of bile acids. Particullary, sulfocholic and trioxocholenoic acids were increased in AF in fetuses who were going to develop brain injury, most of them IVH. Trioxocholenoic acid may be isolated from liver cells. It is chemically very similar to the synthetic compound dehydrocholic acid. Experiments with the bile salt sodium dehydrocholate (DHC) have shown that when injected into the carotid artery, DHC cause extravasation of the albumin-binding dye Evans blue, and it has been proposed as such an in vivo model of blood−brain barrier dysfunction.[42] Kang et al.[43] found thrombosis of the middle cerebral artery and severe damage of the endothelium by the high concentration of the bile salt DHC in the cerebral arteries that might have led to endothelial injury and a consequent thrombosis, which had been previously proposed.[44] Local injection of DHC has demonstrated to cause blood−brain barrier opening and disruption with leakage of blood from the cerebral vasculature into the brain tissue.[45] Therefore, the role of bile acids in general and trioxocholenoic acid in particular in the development of IVH should be further investigated.

The present study has some limitations. The main one is that a substantial proportion of cases have been excluded due to the need to analyze very homogeneous groups. In real life, there are cases of intratuterine infections not confirmed by placental histology and cases of perinatal injury not related to chorioamnionitis but probably to prematurity as those that were excluded for the metabolomics study. Therefore, our conclusions cannot be applied to cases other than those of histological chorioamnionitis and its related perinatal neurological injury. It is known that even in normal pregnancies, placental bacterias have been identified with diverse metabolic and immune regulatory functions.[46] Therefore, potentially bacterial metabolomics may influence the results of the study, and this is another reason to exclude cases with intrauterine infection without demonstrated chorioamnionitis. For the cases included, the influence of bacterial metabolism cannot be evaluated, but in any case, the identified metabolites were so clearly discriminating between cases and controls regardless the type of germen identified that more than probably they came from a maternal, placental, or fetal origin rather than from the bacterial metabolism. In addition, the exclusion of the cases reduced the number of cases fulfilling the strict inclusion criteria. For some analyses, such as differences between cases with or without neurological injury, this lead to a clear need for further studies including greater number of cases to reach definitive conclusions. Finally, validating studies are needed to confirm whether or not the proposed metabolites can be accepted as new biomarkers for the studied conditions.

In conclusion, LC−QTOF-MS is a sensitive tool for metabolomics analysis that may be successfully employed for AF metabolic fingerprinting related to bacterial infection of the fetal amnion and chorionic membranes. Understanding these processes at a molecular level is an important challenge that may provide further insight into the disease pathophysiology and disease onset and allow researchers to obtain metabolomics fingerprint that can help to discover molecules that could serve as potential clinical biomarkers or contribute to the development of new diagnostics strategies for pregnancy related disorders. We would like to highlight the novelty and proposed lactosylceramides and LPE(16:0)/LPE(P-16:0) as candidates for biomarkers of chorioamnionitis. LPC(18:2), sulfocholic acid, and specially trioxocholenoic acid could be consideredas compounds that could have possible diagnostic meaning in case of chorioamnionitis associated with perinatal brain damage. However, further studies with larger numbers of samples are needed to evaluate the predictive capability and potential biomarker implications of these compounds.

ASSOCIATED CONTENT

Supporting Information

Spearman's rank correlation analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AF, amniotic fluid; AUC, area under the curve; C, control group; CH, chorioamnionitis samples without sign of neurological damage; CHN, chorioamnionitis samples with neurological damage; CHT, total number of chorioamnionitis samples; CID, collision-induced dissociation; DNA, deoxyribonucleic acid; Dual AJS ESI, dual agilent jet stream electrospray ionization; ESI(+), electrospray ionization, operated in positive mode; ESI(−), electrospray ionization, operated in negative mode; HMDB, Human Metabolome Database; HP-921, hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine; IL-6, interleukin 6; IVH, intraventricular hemorrhage; KEGG, Kyoto Encyclopedia of Genes and Genomes; LacCer, lactosylceramide; LC−MS, liquid chromatography−mass spectrometry;

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LIPIDMAPS, lipid metabolites and pathways strategy; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; METLIN, Scripps Center for Metabolomics; MFE, molecular feature extraction; MRI, magnetic resonance imaging; NKT, natural killer T-lymphocytes; OCT RF Vpp, octopole radio frequency voltage; OPLS-DA, orthogonal partial least squares discriminant analysis; PC, phosphatidylcholine; PCA-X, principal component analysis; PCR, polymerase chain reaction; PLS-DA, partial least squares discriminant analysis; PNI, perinatal neurological injury; PPROM, preterm premature rupture of membranes; rRNA, ribosomal ribonucleic acid; ROC, receiver−operating characteristic; RSD, relative standard deviation; RT, retention time; SD, standard deviation; SM, sphingomyelin; $TFANH_4$, ammonium trifluoroacetate; TOF, time-of-flight; UHPLC, ultra high-performance liquid chromatography; QC, quality control; LC−QTOF-MS, liquid chromatography−mass spectrometry coupled with quadrupole and time-of-flight mass detector; R^2, variance explained; Q^2, variancepredicted

REFERENCES

(1) Yoon, B. H.; Jun, J. K.; Romero, R.; et al. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. *Am. J. Obstet. Gynecol.* 1997, *177* (1),19−26.

(2) Yoon, B. H.; Romero, R.; Yang, S. H.; et al. Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. *Am. J. Obstet. Gynecol.* 1996, *174* (5), 1433−1440.

(3) O'Shea, T. M. Cerebral palsy in very preterm infants: New epidemiological insights. *Ment. Retard. Dev. Disabil. Res. Rev.* 2002, *8* (3), 135−145.

(4) Yoon, B. H.; Park, C. W.; Chaiworapongsa, T. Intrauterine infection and the development of cerebral palsy. *BJOG* 2003, *110* (Suppl. 20), 124−127.

(5) Bugatto, F.; Fernández-Deudero, A.; Bailén A.; Fernández-Macías, R.; Hervías-Vivancos, B.; Bartha, J. L. Second-trimester amniotic fluid proinflammatory cytokine levels in normal and overweight women. *Obstet. Gynecol.* 2010, *115* (1),127−133.

(6) Romero, R.; Mazaki-Tovi, S.; Vaisbuch, E.; et al. Metabolomics in premature labor: A novel approach to identify patients at risk for preterm delivery. *J. Matern.-Fetal Neonat. Med.* 2010, *23* (12), 1344−1359.

(7) Yang, S.; Lin, S.; Kelen, G. D.; et al. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *J. Clin. Microbiol.* 2002, *40* (9), 3449−3454.

(8) Romero Gómez, M. P.; García-Perea, A.; Ruiz Carrascoso, G.; Bajo, M. A.; Mingorance, J. Campylobacter fetus peritonitis and bacteremia in a patient undergoing continuous ambulatory peritoneal dialysis. *J. Clin. Microbiol.* 2010, *48* (1),336−337.

(9) Ciborowski, M.; Javier Ruperez, F.; Martinez-Alcazar, M. P.; et al. Metabolomic approach with LC−MS reveals significant effect of pressure on diver's plasma. *J. Proteome Res.* 2010, *9* (8), 4131−4137.

(10) Gika, H. G.; Macpherson, E.; Theodoridis, G. A.; Wilson, I. D. Evaluation of the repeatability of ultra-performance liquid chromatography−TOF-MS for global metabolic profiling of human urine samples. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* 2008, *871* (2), 299−305.

(11) Xia, J.; Mandal, R.; Sinelnikov, I. V.; Broadhurst, D.; Wishart, D. S. MetaboAnalyst 2.0—a comprehensive server for metabolomic data analysis. *Nucleic Acids Res.* 2012, *40* (Web Server issue), W127−133.

(12) Xia, J.; Broadhurst, D. I.; Wilson, M.; Wishart, D. S. Translational biomarker discovery in clinical metabolomics: An introductory tutorial. *Metabolomics* 2013, *9* (2),280−299.

(13) Westerhuis, J.; Hoefsloot, H.; Smit, S.; et al. Assessment of PLSDA cross validation. *Metabolomics* 2008, *4* (1),81−89.

(14) Kamath-Rayne, B. D.; Smith, H. C.; Muglia, L. J.; Morrow, A. L. Amniotic fluid: The use of high-dimensional biology to understand fetal well-being. *Reprod. Sci.* 2014, *21* (1),6−19.

(15) Zheng, W.; Kollmeyer, J.; Symolon, H.; et al. Ceramides and other bioactive sphingolipid backbones in health and disease: Lipidomic analysis, metabolism, and roles in membrane structure, dynamics, signaling, and autophagy. *Biochim. Biophys. Acta* 2006, *1758* (12), 1864−1884.

(16) Assi, E.; Cazzato, D.; De Palma, C.; Perrotta, C.; Clementi, E.; Cervia, D. Sphingolipids and brain resident macrophages in neuroinflammation: An emerging aspect of nervous system pathology. *Clin. Dev. Immunol.* 2013, *2013*, 309302.

(17) Maceyka, M.; Spiegel, S. Sphingolipid metabolites in inflammatory disease. *Nature* 2014, *510* (7503), 58−67.

(18) Chatterjee, S.; Pandey, A. The Yin and Yang of lactosylceramide metabolism: Implications in cell function. *Biochim. Biophys. Acta* 2008, *1780* (3), 370−382.

(19) Bhunia, A. K.; Arai, T.; Bulkley, G.; Chatterjee, S. Lactosylceramide mediates tumor necrosis factor-alpha-induced intercellular adhesion molecule-1 (ICAM-1) expression and the adhesion of neutrophil in human umbilical vein endothelial cells. *J. Biol. Chem.* 1998, *273* (51), 34349−34357.

(20) Moore, R. M.; Silver, R. J.; Moore, J. J. Physiological apoptotic agents have different effects upon human amnion epithelial and mesenchymal cells. *Placenta* 2003, *24* (2−3), 173−180.

(21) Hallman, M.; Bry, K.; Pitkäen, O. Ceramide lactoside in amniotic fluid: High concentration in chorioamnionitis and in preterm labor. *Am. J. Obstet. Gynecol.* 1989, *161* (2), 313−318.

(22) Arai, T.; Bhunia, A. K.; Chatterjee, S.; Bulkley, G. B. Lactosylceramide stimulates human neutrophils to upregulate Mac-1, adhere to endothelium, and generate reactive oxygen metabolites in vitro. *Circ. Res.* 1998, *82* (5), 540−547.

(23) Won, J. S.; Singh, A. K.; Singh, I. Lactosylceramide: A lipid second messenger in neuroinflammatory disease. *J. Neurochem.* 2007, *103* (Suppl. 1),180−191.

(24) Pannu, R.; Singh, A. K.; Singh, I. A novel role of lactosylceramide in the regulation of tumor necrosis factor alpha-mediated proliferation of rat primary astrocytes. Implications for astrogliosis following neurotrauma. *J. Biol. Chem.* 2005, *280* (14), 13742−13751.

(25) Dong, J.; Cai, X.; Zhao, L.; et al. Lysophosphatidylcholine profiling of plasma: Discrimination of isomers and discovery of lung cancer biomarkers. *Metabolomics* 2010, *6* (4),478−488.

(26) D'Arrigo, P.; Scotti, M. Lysophospholipids: Synthesis and biological aspects. *Curr. Org. Chem.* 2013, *17* (8),812−830(819).

(27) Dudzik, D.; Zorawski, M.; Skotnicki, M.; et al. Metabolic fingerprint of gestational diabetes mellitus. *J. Proteomics* 2014, *103*, 57−71.

(28) Floegel, A.; Stefan, N.; Yu, Z.; et al. Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. *Diabetes* 2013, *62* (2), 639−648.

(29) Del Boccio, P.; Pieragostino, D.; Di Ioia, M.; et al. Lipidomic investigations for the characterization of circulating serum lipids in multiple sclerosis. *J. Proteomics* 2011, *74* (12),2826−2836.

(30) Asaoka, Y.; Oka, M.; Yoshida, K.; Sasaki, Y.; Nishizuka, Y. Role of lysophosphatidylcholine in T-lymphocyte activation: Involvement of phospholipase A2 in signal transduction through protein kinase C. *Proc. Natl. Acad. Sci. U. S. A.* 1992, *89* (14), 6447−6451.

(31) Fox, L. M.; Cox, D. G.; Lockridge, J. L.; et al. Recognition of lysophospholipids by human natural killer T -lymphocytes. *PLoS Biol.* 2009, *7* (10),e1000228.

(32) Sevastou, I.; Kaffe, E.; Mouratis, M. A.; Aidinis, V. Lysoglycerophospholipids in chronic inflammatory disorders: The PLA(2)/LPC and ATX/LPA axes. *Biochim. Biophys. Acta* 2013, *1831* (1), 42−60.

(33) Stock, J. The emerging role of lipidomics. *Atherosclerosis* 2012, *221* (1), 38−40.

(34) Tan, Y.; Yin, P.; Tang, L.; et al. Metabolomics study of stepwise hepatocarcinogenesis from the model rats to patients: Potential

biomarkers effective for smallhepatocellular carcinomadiagnosis. *Mol. Cell Proteomics* 2012, *11* (2), M111.010694.
(35) Frasch, S. C.; Bratton, D. L. Emerging roles for lysophosphatidylserine in resolution of inflammation. *Prog. Lipid Res.* 2012, *51* (3), 199−207.
(36) Menon, R.; Jones, J.; Gunst, P. R.; et al. Amniotic fluid metabolomic analysis in spontaneous preterm birth. *Reprod. Sci.* 2014, *21* (6), 791−803.
(37) Abehsera, D.; Rodrigues, Y.; Mingorance, J.; Suárez, A.; Magdaleno, F.; Bartha, J. L. Prediction and clinical relevance of pathologicpatternsofinjury associatedwith chorioamnionitis. *Placenta* 2014, *35* (1), 70−71.
(38) Perez, M. J.; Velasco, E.; Monte, M. J.; Gonzalez-Buitrago, J. M.; Marin, J. J. Maternal ethanol consumption during pregnancy enhances bile acid-induced oxidative stress and apoptosis in fetal rat liver. *Toxicology* 2006, *225* (2−3), 183−194.
(39) Perez, M. J.; Macias, R. I.; Duran, C.; Monte, M. J.; Gonzalez-Buitrago, J. M.; Marin, J. J. Oxidative stress and apoptosis in fetal rat liver induced by maternal cholestasis. Protective effect of ursodeoxycholic acid. *J. Hepatol.* 2005, *43* (2), 324−332.
(40) Perez, M. J.; Briz, O. Bile-acid-induced cell injury and protection. *World J. Gastroenterol.* 2009, *15* (14), 1677−1689.
(41) Amaral, J. D.; Viana, R. J.; Ramalho, R. M.; Steer, C. J.; Rodrigues, C. M. Bile acids: Regulation of apoptosis by ursodeoxycholic acid. *J. Lipid Res.* 2009, *50* (9), 1721−1734.
(42) Spigelman, M. K.; Zappulla, R. A.; Malis, L. I.; Holland, J. F.; Goldsmith, S. J.; Goldberg, J. D. Intracarotid dehydrocholate infusion: A new method for prolonged reversible blood−brain barrier disruption. *Neurosurgery* 1983, *12* (6), 606−612.
(43) Kang, E. J.; Major, S.; Jorks, D.; et al. Blood−brain barrier opening to large molecules does not imply blood−brain barrier opening to small ions. *Neurobiol. Dis.* 2013, *52*, 204−218.
(44) Lossinsky, A. S.; Vorbrodt, A. W.; Wisniewski, H. M. Scanning and transmission electron microscopic studies of microvascular pathology in the osmotically impaired blood−brain barrier. *J. Neurocytol.* 1995, *24* (10), 795−806.
(45) Jorks, D.; Milakara, D.; Alam, M.; et al. A novel algorithm for the assessment of blood−brain barrier permeability suggests that brain topical application of endothelin-1 does not cause early opening of the barrier in rats. *Cardiovasc. Psychiatry Neurol.* 2011, *2011*, 169580.
(46) Aagaard, K. M. Author response to comment on "the placenta harbors a unique microbiome". *Sci. Transl. Med.* 2014, *6* (254), 254lr253.

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ORIGINAL ARTICLE

Prediction of chorioamnionitis in cases of intraamniotic infection by ureaplasma urealyticum in women with very preterm premature rupture of membranes or preterm labour

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ABSTRACT

Objectives: First, to determinate the frequency of chorioamnionitis and funisitis in cases of intramniotic detection of Ureaplasma urealyticum. Second, to assess the predictive capability of some biological markers in the amniotic fluid of these women to predict histological inflammation.

Subjects and methods: We prospectively studied 20 cases of women with premature rupture of membranes or preterm labour (PROM) or preterm labour and intraamniotic detection of Ureaplasma urealyticum. Gestational age at admission was 26.74 ± 2.53 weeks. Amniotic fluid concentrations of IL18, IL 2, IL4, IL6, IL10, IL12, TNF-alpha, IFN-g, and MMP-8 were measured by the Multiplex method. Amniotic fluid glucose and leukocyte count were also measured by standard methods. Placental detailed histological studies were performed. Student's *t*-test, forward stepwise conditional binary logistic regression analysis and ROC curves were used.

Results: Histological chorioamnionitis was present in 45% of cases (9/20) and funisitis just in 15% (3/20). Interleukins 6, 8, 12, MMP-8, and leukocyte count were significantly elevated in cases of histological inflammation, defined as choriamnionitis or chorioamniotis þ funisitis (p ¼ .007, .03, .01, .03, .03, respectively) while glucose was decreased (p ¼ .04). Binary logistic regression for the prediction of inflammation showed a high predictive value (R^2 ¼ .66, p ¼ .002) including in the equation only the IL6 value.

Conclusions: A significant percentage of cases with intraamniotic detection of Ureaplasma urealyticum shows no pathological signs of histological inflammation. Concentration of Interleukin 6 in amniotic fluid can be useful for the diagnosis of subclinical chorioamnionitis in these cases.

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Introduction

In recent years, the fundamental role of intra-amniotic inflammation in the development of preterm labour has become evident [1,2]. It should be considered, however, that not all intra-amniotic infections will lead to an inflammatory process extending to the foetal component [3–5]. Inflammation that occurs in the setting of an intrauterine infection varies in degree and it is a variable phenomenon, probably with different levels of intensity from mild processes with limited consequences to a state that predisposes to severe brain damage or even foetal death [6–8].

Ureaplasma urealyticum is the bacterial species most often found in amniotic fluid (AF). Up to 35% cases of intraamniotic infection will be caused by Ureaplasma [3,9]. The earlier the gestational age, the more likely these organisms are to be present in the AF, the placenta or in the membranes [9]. However, it can colonise the choriodecidua and the AF without causing inflammation. Consequently, it causes preterm birth in less than 25% of cases while most of pregnancies may proceed normally to term [10,11].

This could be due to the immunosuppressive properties of choriodecidua and the low pathogenicity of the microorganism, therefore small amounts of Ureaplasma may not be capable of inducing inflammation in an environment with powerful anti-inflammatory capacity such as decidua [12].

It is also known that poly-microbial colonisation of the amniotic cavity is common in pre-term deliveries, with around half of all infected AF containing two or more microorganisms [13]. Co-colonisation of the AF with Ureaplasma and another genital microorganism has been reported to be associated with more severe

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adverse pregnancy outcomes compared to colonisation with Ureaplasma alone [14].

Thus, the clinical relevance of the presence of Ureaplasma species in the AF, and the development of intra-amniotic inflammatory response appears to be microbial type-, dose-, and gestational age-dependent [15,16]. Regarding the treatment of such infections, Erythromycin was the drug of choice for eradicating Ureaplasma during pregnancy, However, there is little agreement about its effectiveness for treating intra-amniotic infections. Recently, several animal studies have reported that maternal intravenous Azithryomycin can effectively eradicate intra-amniotic Ureaplasma infection [17–20].

Giving the fact that, Ureaplasma is the microorganism most often isolated in cases of intrauterine infection, it is essential to differentiate between the different inflammatory states at intrauterine level to offer a more specific prognosis of neonatal outcomes to guide decisions on what attitude to take regarding the management of the pregnancy.

The objectives of this study are first, to determinate the frequency of chorioamnionitis and funisitis among cases of Ureaplasma intrauterine infection and second, to assess the predictive capability of some biological markers in the AF of women with an intrauterine infection of Ureaplasma to detect histological inflammation, defined as chorioamnionitis alone or chorioamnionitis þ funisitis. We also examine the potential impact of Azitromicin therapy in the inflammatory response of these pregnancies.

Methods

We prospectively studied 20 cases of intraamniotic infection of Ureaplasma urealityum in women with PROM (gestational age between 24 and 32 weeks) or preterm labour with poor prognosis (gestational age between 24 and 28 weeks, refractory to tocolysis, prolapsed amniotic sac in vagina, and/or vaginal bleeding from uterine origin of unknown cause). The exclusion criteria were: multiple gestations, polimicrobial infection detected in AF and/or the presence of clinical chorioamnionitis.

PROM was diagnosed by the presence of AF leakage in the vagina and/or by using a test detecting IGFBP-1 (insulin-like growth factor binding protein-1) in vaginal samples in the presence of olygohydramnios. Preterm labour was diagnosed by the presence of at least two regular uterine contractions every 10 min associated with cervical length <15 mm that required hospital admission and tocolytic treatment. Women with the diagnosis of PROM were treated after Amniocentesis with Ampicillin 1 g IV/6 h þGentamicin 240 mg/24 h þ Azitromicina 1 g oral/72 h during one week, according with local protocols [21].

Ureaplasma intramaniotic infection was detected by diagnostic amniocentesis, universal and specific PCR, and microbiological cultures at the moment of admission. An extra 3 ml of AF for the study was obtained after the patient gave her written informed consent. After amniocentesis, the AF was immediately transported in a capped sterile syringe to the biobank, and stored at $-80\,^{o}C$ until analysis. The study was approved by the local Ethical and Research Committees, and all the women in the study signed a written consent form for their participation. All the samples were collected in a single institution

A molecular analysis was performed as follows: Total DNA was extracted from 400 1L of AF sample and eluted in 100 1l of elution buffer using a MagNA Pure Compact system and the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics GmbH, Mannheim, Germany). Detection of bacterial DNA was done by broad-range real-time PCR, targeting the 16 S rRNA gene using Takara Premix Ex Taq and oligonucleotides P891F, P1033R, and the Taqman probe Uniprobe7. Specific assays were developed by substituting the forward primer by primers ChlaF 5^{0}-GTATGCCGCCTGAGGAGTACA-3^{0} for Chlamydia sp., MychF 5^{0}-GCCTGAGTAGTATGCTCGCAAGA-3^{0} for Mycoplasma sp., and UreF 5^{0}-GCCTGGGTAGTACATTC GCAAGA-3^{0}for Ureaplasma sp. Each sample was tested in parallel with the universal and the three specific sets. Negative and positive controls were included in each assay. All the positive samples were identified by amplification and pyrosequencing of three short regions of the 16 S rRNA gene as described elsewhere [22].

The AF processing for culturing aerobic and anaerobic bacteria was performed as follows: The sample was centrifuged, the supernatant discarded, and the product planted onto four different media. Gram staining was performed for the direct examination of Gram-positive bacteria. The growth media used were: blood agar (in a CO_2 incubator), chocolate agar (also in a CO_2 incubator), blood agar enriched with vitamin K1 and haemin (anaerobic incubation) for anaerobic microorganisms, and plating liquid (thioglycolate). The samples were evaluated every 24 h, except that, when the planting was performed anaerobically, the initial evaluation was at 48 h. The culture medium used for the detection of urogenital mycoplasmas is a commercial medium "Mycofast Evolution 2" (ELITech France SAS). Only the samples where an infection by Ureaplasma Urealyticum alone was detected were included in the study.

The gross examination of the placenta in the Department of Pathology included: weight and other standard measurements; colour, appearance, and integrity of membranes; description of any gross lesion; and length, colour, insertion, coiling. and vessels in the umbilical cord. The histological study included: three sections of cord (proximal, middle, and distal to the placenta); one membrane roll (by the "jelly roll" method in order to obtain a maximum amount of membranes with decidua capsularis); and three full thickness sections of parenchyma, including chorionic plate vessels. Tissues were fixed in 10% formalin solution for at least 6 h before paraffin embedding, followed by haematoxylin and eosin staining.

Histological chorioamnionitis and funisitis were classified according to the severity of the injury in the following specific pathologic patterns according to Redline classification [23]: (A) Chorioamnionitis: early acute subchorionitis/chorionitis, acute chorioamnionitis and necrotising chorioamnionitis and (B) Funisitis: umbilical phlebitis/chorionic vasculitis, umbilical arteritis and concentric periphlebitis/necrotising.

The cytokines TNF-alpha/IFN-gamma/IL-1b/IL-2/IL-6/IL-8/IL-12/IL-4/IL-10/MMP-8 were measured in AF using a multiple immunoassay kit with magnetic beads (Immunoassay Kit Magnetic Plex Human 10) of Affymetrix, following the manufacturer's instructions. The plates consisted of 96 wells, 16 used to perform the calibration curve from eight standards processed in duplicate, two used for the blank, and the remain- ing 78 wells for samples of AF (25 1L) done in duplicate. The quantitative readout was conducted on a Luminex-200 xMap Technology® (France).

The results were analysed using the SPSS 22.0 software package (SPSS, Chicago, IL). The distribution of variables was assessed by analysing the histograms. Since most of the variables are distributed non-parametrically, most of values are presented as median and interquartile range. On the contrary, gestational age and maternal age are presented as mean and standard deviations. Comparison between groups was performed using the Kruskal–Wallis' test. The *post-hoc* analysis comparing paired groups was performed with DNS test. Prediction for the presence of chorioamnionitis was estimated by forward stepwise conditional binary logistic regression analysis and ROC curves. The pre-set level of significance was 95% ($p < .05$).

Results

From the total of 20 women included in the study, three (15%) were cases of preterm labour with intact membranes and poor prognosis as above described and 17 (85%) were cases of PPROM. Maternal age was 32.63 ± 5.61 years; 15 were nulliparous (75%). The mean gestational age at admission was 26.74 ± 2.53 weeks and the mean gestational age at delivery was 27.94 ± 2.34 weeks with an interval time between admission and delivery of 8.46 days.

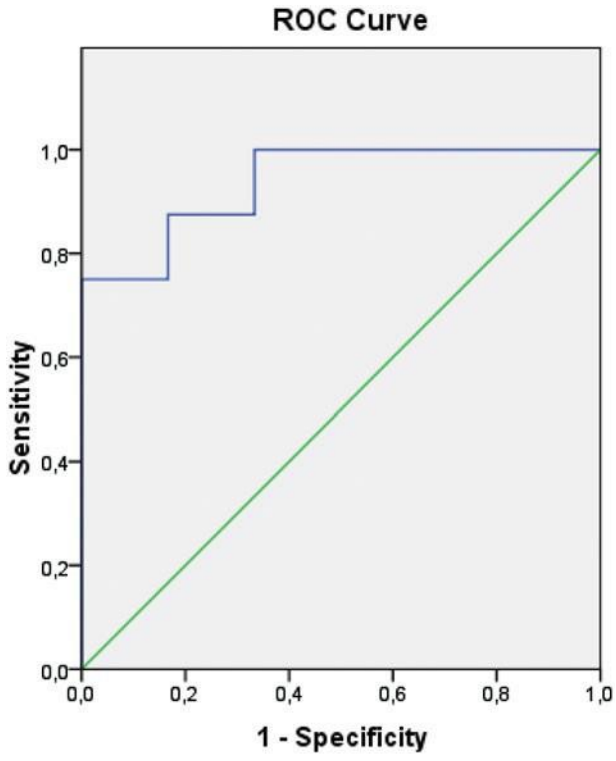
PCR analysis identified all cases of intraamniotic infection. In six out of the 20 cases, the microbiological culture was not performed due to the small amount of AF. All these six cases were patients with PPROM with reduced AF. The microbiological culture showed infection in just two of the 14 samples analysed (14%).

Of the 20 cases with intra-amniotic infection, we found eight cases with no histological lesions (40%). The 12 cases in which placental histological lesions were demonstrated, consisted in chorioamnionitis alone (9/20, 45%) and chorioamnionitis associated with funisitis (3/20, 15%). There was no case of funisitis without chorioamnionitis. The two cases where the microbiological culture was positive developed chorioamnionitis without funisitis.

For the prediction of inflammation, we used the cases of choriamnionitis and chorioamnionitis þ funisitis. Predictors of histological inflammation defined as chorioamnionitis alone or chorioamnionitis þfunisitis were analysed by using linear regression and ROC curves. Clinical parameters such as maternal characteristics, gestational age, leukocytes count in AF and biochemical markers including the cytokines TNF-alpha/IFN-gamma/IL-1b/IL-2/IL-6/IL-8/IL-12/IL-4/IL-10/MMP-8 wereincluded.

Interleukins 6, 8, 12, MMP-8, and leukocyte count were significantly elevated in cases of histological inflammation (p ¼ .007, .03, .01, .03, .03, respectively) while glucose was decreased (p ¼ .04). Binary logistic regression for the prediction of inflammation showed a high predictive value (R^2 ¼ .66, p ¼ .002) including in the equation only the IL6 value (Figure 1). If cytokine measurements were excluded from the analysis, the best predictive model included only glucose levels but the predictive ability decreased (R^2 ¼ .43, p ¼ .02). Cut-off values of IL6 in amniotic fluid would range between 400 and 900 pg/mL depending on the required sensitivity and specificity (100% and 66% in the first cut-off versus 75% and 100% in the second one).

As said before, from the 20 patients studied, 17 were cases of PROM and all of them but one were treated with regime of antibiotics approved by local protocols. One of the cases of PROM were treated with a regime of Ampicilin and Gentacimin alone because the admission date was before we included



AUC 0.93 (CI 95% 0.00 – 1.00) (p = 0.002).

Figure 1. ROC curve for binary logistic regression for the prediction of inflammation including IL 6.

Azitromicin in the protocol. Three cases of the study were preterm labour without PROM and therefore, they were treated with a course of Atosiban without any antibiotic. From the three cases of funisitis identified, two of them were preterm labour without PROM and one of them was the case of PROM treated with Ampicilin and Gentacimicin alone. Thus, from the 16 cases of the studied treated with Azitromicin, half of them developed chorioamnitis and the other half presented with no histological placental lesion. However, none of the cases treated with Azitromicin developed funisitis.

Discussion

Ureaplasma urealyticum is commonly found in amniotic fluid and membranes in association with PROM and preterm labour. Goldenberg et al. [9] studied the cases of Ureaplasma urealyticum or Mycoplasma hominis or both in cord blood of preterm infants delivered at 23–32 weeks. They found that a positive culture for Ureaplasma urealyticum or Mycoplasma hominis or both was present in 82 of the 351 cases studied (23.4%), However, a positive culture for Ureaplasma urealyticum alone was present in 43 out of the 351 (12%). Therefore, according to this report, just about 12% of all preterm infants born before 32 weeks will have a positive umbilical cord culture for Ureaplasma. Furthermore, it has been shown that even in the presence of a high microbial load of this bacteria in the umbilical cord in pregnancies complicated by PROM, this is not associated with high foetal inflammatory response [16].

However, the proportion of intrauterine infections caused by Ureaplasma that develops a histological chorioamnionitis and funisitis remains unknown. The results from this study show that the vast majority of intrauterine infections by Ureaplasma are not associated with funisitis. From the 20 patients evaluated, up to 40% did not develop any inflammatory histological lesion. 45% of patients with intrauterine infection evolved to histological chorioamnionitis, however, just in 15% of the cases the inflammation reached the foetal component leading the funisitis.

Bacterial infection that induces an inflammatory response has been shown to be a crucial factor for the onset of preterm labour [24]. However, colonisation without inflammation appears relatively benign and intrauterine inflammation is not simply present or absent but also has degrees of severity that correlate with adverse outcomes [4]. Histological inflammation with increased levels of pro-inflammatory cytokines is better correlated with a foetal inflammatory response syndrome than presence of bacteria in the same compartment [25,26]. Therefore, we evaluated a profile of cytokines and biological markers that would be predictive for the detection of chorioamnionitis in patients with Ureaplasma intra-amniotic infection.

From all the studied cytokines (TNF-alpha/IFN-gamma/IL-1b/IL-2/IL-6/IL-8/IL-12/IL-4/IL-10, and MMP-8) just IL 6, 8, 12, and MMP8 were significantly elevated in cases of histological inflammation. However, we found no correlation between choriamnionitis or chorioamninitis and funisitis and TNF-alpha, IFN-gamma, L-1b, IL-2, IL4 or IL-10. A decreased in the intraamni- otic glucose level and an increased in leucocyte count were also related with inflammation.

We also performed a binary logistic regression in other to identify the best predictor of histological inflammation in cases of Ureaplasma infection. IL 6 showed a high predictive value for a cut off between 400 and 900 pg/mL. With an IL6 cut off of 400 pg/ml, we will obtain a detection rate of chorioamnionitis of 100% with a specificity of 66%. On the other hand, for a cut off of 900 pg/ml we will increase the specificity up to 100% with a detection rate of 75%. Therefore, IL 6 alone, rather than a combination of several cytokines appears to be the best predictor of histological inflammation in cases of Ureaplasma infection.

Several previous reports have addressed the relationship between pro inflammatory cytokines and subclinical chorioamnionitis and foetal inflammatory response syndrome. However, there are few articles studying the relationship between Ureaplasma intra-amniotic infection with proinflamatory cytokines. Yoon et al. [27] based the diagnosis of inflammatory response in women in Ureaplasma infection in the presence of high levels of IL 6 and Yoneda et al. [28] found high AF IL8 levels in these patients compared with those in microorganism-negative cases. Jacobson et al. [15] reported a significant correlation between Ureaplasma DNA and TNF-a. They did not find any correlation with other IL such as interleukin IL-6, IL-1 b, and IL-10. However, to our knowledge this is the first study evaluating Ureaplasma infections with a panel of several biological markers and the combination of them, for the diagnosis of histological inflammation.

In a previous report from our group [3], we evaluated the same profile of biological markers in a cohort of cases at high risk of intrauterine infection. We found that, regardless the microorganism involved, IL 6, 8, and 12 were capable by itself to identify the presence of inflammatory lesions in cases of intra-amniotic infection and to distinguish between chorioamnionitis and choriomanionitis plus funisitis.

The results from this study which just evaluates Ureaplasma intrauterine infection, are consistent with the previous findings. Therefore, it appears that could be the level of any of these pro inflammatory cytokines the tool we should use to identify those patients with an inflammatory process and at risk of foetal inflammatory response syndrome despite the microorganism causing the infection.

Although antibiotic therapy is standard of care for preterm premature rupture of membranes management, usual antibiotic regimens such as Erythromicin have a limited transplacental transfer and fail to eradicate intraamniotic Ureaplasma or to improve neonatal outcome. Several animal models [17–19] have reported that maternal intravenous Azytromicin can effectively eradicate intraamniotic Ureaplasma, prolong pregnancy and reduce neonatal morbidity, although in this previous report the authors could not demonstrate a reduction in acute chorioamnionitis.

In this study, we treated 16 out of the 17 patients with the diagnosis of PROM with a regime of Ampicilin 1 g IV/6 h þ Gentamicin 240/24 h þ Azitromicin 1 g oral/72 h. From these cases, none of them developed histological funisitis and half of them presented with no histological placental lesion. From the three cases of our study with funisitis none of them received Azitromicin therapy.

However, there are mayor differences between this regimen and the one published before. Grigsby et al. [17] used multiple azithromycin intravenous injections whereas we prescribed oral Azytromicin and with different doses from the ones published before. Furthermore, the number of cases is still limited to draw any conclusion to thisrespect.

In summary, intraamniotic infection with Ureaplasma in women with preterm labour or PROM does not necessarily lead to intrauterine inflammation, actually 40% will not develop histological lesions. In order to identify those pregnancies that will present chorioamnionitis, IL 6, 8, 12, and MMP8 were significantly elevated in cases of histological inflammation and an AF IL6 cut off of 400 pg/ml will detect chorioamnionitis in a 100% of cases with a specificity of 66%. Azytromicin is a promising therapy is cases of Ureaplasma intraamniotic infection, however further studies are needed to conclude its effectiveness and to establish the best treatment approach.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] Goldenberg RL, Culhane JF, Iams JD, et al. Epidemiology and causes of preterm birth. Lancet. 2008;371:75–84.

[2] Romero R, Espinoza J, Goncalves LF, et al. The role of inflammation and infection in preterm birth. Semin Reprod Med. 2007;25:21–39.

[3] Revello R, Alcaide MJ, Dudzik D, et al. Differential amniotic fluid cytokine profile in women with chorioamnionitis with and without funisitis. J Matern Fetal Neonatal Med. 2016;29:2161–2165.

[4] Combs CA, Gravett M, Garite TJ, et al. Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. Am J Obstet Gynecol. 2014;210:125.

[5] Ireland DJ, Keelan JA. The maternal serological response to intrauterine Ureaplasma sp. infection and prediction of risk of pre-term birth. Front Immunol. 2014;9:624.

[6] Pacora P, Chaiworapongsa T, Maymon E, et al. Funisitis and chorionic vasculitis: the histological counterpart of the fetal inflammatory response syndrome. J Matern Fetal Neonatal Med. 2002;11:18–25.

[7] Sampson JE, Theve RP, Blatman RN, et al. Fetal origin of amniotic fluid polymorphonuclear leukocytes. Am J Obstet Gynecol. 1997;176:77–81.

[8] Romero R, Mazor M. Infection and preterm labor. Clin Obstet Gynecol. 1988;31:553–584.
[9] Goldenberg RL, Andrews WW, Goepfert AR, et al. The alabama preterm birth study: umbilical cord blood Ureaplasma urealyticum and Mycoplasma hominis cultures in very preterm newborn infants. Am J Obstet Gynecol. 2008;198:43.
[10] Gerber S, Vial Y, Hohlfeld P, et al. Detection of Ureaplasma urealyticum in second-trimester amniotic fluid by polymerase chain reaction correlates with subsequent preterm labor and delivery. J Infect Dis. 2003;187:518–521.
[11] Perni SC, Vardhana S, Korneeva I, et al. Mycoplasma hominis and Ureaplasma urealyticum in midtrimester amniotic fluid: association with amniotic fluid cytokine levels and pregnancy outcome. Am J Obstet Gynecol. 2004;191:1382–1386.
[12] Aaltonen R, Heikkinen J, Vahlberg T, et al. Local inflammatory response in choriodecidua induced by Ureaplasma urealyticum. BJOG. 2007;114:1432–1435.
[13] DiGiulio DB. Diversity of microbes in amniotic fluid. Semin Fetal Neonatal Med. 2012;17:2–11.
[14] Kwak DW, Hwang HS, Kwon JY, et al. Co-infection with vaginal Ureaplasma urealyticum and Mycoplasma hominis increases adverse pregnancy outcomes in patients with preterm labor or preterm premature rupture of membranes. J Matern Fetal Neonatal Med. 2014;27:333–337.
[15] Jacobsson B, Aaltonen R, Rantakokko-Jalava K, et al. Quantification of Ureaplasma urealyticum DNA in the amniotic fluid from patients in PTL and pPROM and its relation to inflammatory cytokine levels. Acta Obstet Gynecol Scand. 2009;88:63–70.
[16] Kacerovsky M, Pliskova L, Menon R, et al. Microbial load of umbilical cord blood Ureaplasma species and Mycoplasma hominis in preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2014;27:1627–1632.
[17] Grigsby PL, Novy MJ, Sadowsky DW, et al. Maternal azithromycin therapy for Ureaplasma intraamniotic infection delays preterm delivery and reduces fetal lung injury in a primate model. Am J Obstet Gynecol. 2012;207:475.
[18] Miura Y, Payne MS, Keelan JA, et al. Maternal intravenous treatment with either azithromycin or solithromycin clears Ureaplasma parvum from the amniotic fluid in an ovine model of intrauterine infection. Antimicrob Agents Chemother. 2014;58:5413–5420.
[19] Acosta EP, Grigsby PL, Larson KB, et al. Transplacental transfer of Azithromycin and its use for eradicating intra-amniotic ureaplasma infection in a primate model. J Infect Dis. 2014;209:898–890.
[20] Lee SJ, Ahn JW, Lee JY, et al. Maternal azithromycin administration eradicates intra-amniotic Ureaplasma infection: the first human case report. J Obstet Gynaecol. 2016;36:259–260.
[21] Protocolos SEGO: Rotura prematura de membranas [Internet]. [cited 2016 November]. Available from: http://www.prosego.com.
[22] Romero Gomez MP, Garc ıa-Perea A, Ruiz Carrascoso G, et al. Campylobacter fetus peritonitis and bacteremia in a patient undergoing continuous ambulatory peritoneal dialysis. J Clin Microbiol. 2010;48:336–337.
[23] Redline RW. Inflammatory response in acute chorioamnionitis. Semin Fetal Neonatal Med. 2012;17:20–25.
[24] Dudley DJ. Pre-term labor: an intra-uterine inflammatory response syndrome? J Reprod Immunol. 1997;36:93–109.
[25] Shim SS, Romero R, Hong JS, et al. Clinical significance of intra-amniotic inflammation in patients with preterm premature rupture of membranes. Am J Obstet Gynecol. 2004;191:1339–1345.
[26] Jacobsson B, Mattsby-Baltzer I, Andersch B, et al. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women in preterm labor. Acta Obstet Gynecol Scand. 2003;82:120–128.
[27] Yoon BH, Romero R, Lim JH, et al. The clinical significance of detecting Ureaplasma urealyticum by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. Am J Obstet Gynecol. 2003;189:919–924.
[28] Yoneda N, Yoneda S, Niimi H. Polymicrobial amniotic fluid infection with Mycoplasma/Ureaplasma and other bacteria induces severe intra-amniotic inflammation associated with poor perinatal prognosis in preterm labor. Am J Reprod Immunol. 2016;75: 112–125.

4.2 RESULTADOS GLOBALES

ESTUDIO 1

El estudio incluyó 40 pacientes con riesgo de infección intraamniótica. 12 (30%) fueron casos de APP con membranas íntegras y 28 (70%) RPM pretérmino.

De estas 40 pacientes, 18 (45%) fueron diagnosticadas de infección intraamniótica mediante técnica de PCR. El microorganismo más frecuentemente aislado fue *Ureaplasma urealyticum* (14/40, 35%). De estos 18 casos con infección, 15 (83%) presentaron algún tipo de inflamación histológica, de las cuales sólo el 33% llegaron a progresar a funisitis.

En el estudio anatomopatológico de las placentas analizadas, en 25 de las 40 no se encontraron lesiones inflamatorias (62.5%). De estas 25 placentas sin lesiones, 19 no tenían diagnóstico de infección intraamniótica y en las 6 restantes se evidenció infección por *Ureaplasma urealyticum* mediante técnica de PCR.

De las 15 placentas restantes con lesión histológica, 9 de 40 (22.5%) fueron corioamnionitis aislada y 6 de 40 (15%) corioamnionitis más funisitis.

Se llevó a cabo un análisis estadístico para analizar los niveles de diferentes citoquinas inflamatorias en líquido amniótico (IL-1b, IL-2, IL-4, IL-6, IL 8, IL-10, IL-12, TNF-alpha, IFN-gamma, y MMP-8) de estas pacientes. Se encontraron diferencias estadísticamente significativas entre los grupos de pacientes que desarrollaron lesión histológica comparado con aquellos casos sin lesión, para todos los marcadores analizados, exceptuando IFN-gamma en la que la diferencia fue límite ($p = 0.05$)

En el estudio “post-hoc” grupo a grupo se realizó 3 tipos de comparaciones:

- Comparación entre el grupo sin lesión histológica (grupo control) con elgrupo de corioamnionitis aislada.
- Comparación entre el grupo control con el grupo de corioamnionitis +funisitis.
- Comparación entre el grupo de corioamnionitis aislada con corioamnionitis+ funisitis.

Se realizaron las 3 comparaciones para cada una de las citoquinas estudiadas.

Sólo IL-6, IL-12, IL-8, and MMP-8 presentación diferencias estadísticamente significativas en la comparación entre grupo control y corioamnionitis aislada. Sin embargo, cuando se realizó la comparación entre grupo control y el grupo de corioamnionitis + funisitis se encontraron diferencias en la mayoría de las citoquinas estudiadas: IL-1b, IL-2, IL-6, IL-10, IL-12, IL-8, TNF-alpha, y MMP-8.

Se encontraron diferencias estadísticamente significativas en las citoquinas: IL-1b, IL-6, IL-10, IL-12, IL-8, y TNF-alpha cuando se comparó los grupo de corioamnionitis aislada y corioamnionitis + funisitis.

El análisis de regresión logística binaria aportó un modelo de predicción de funisitis con un alto valor predictivo ($R2= 1$, $p < 0.00001$) y que incluía en la ecuación las citoquinas IL-4, IL-8, IL-10, and IL-12.

ESTUDIO 2

En este trabajo se estudió la misma población que el estudio previo, en la que se evidenció 15 casos de corioamnionitis histológica (37.5%) de las 40 pacientes incluidas, y 18 casos (45%) de infección intraamniótica.

En este caso se analizaron otras variables relacionadas con el resultado perinatal:

- La edad gestacional al parto fue 30.4 ± 4.1 semanas. Esta edad gestacional fue significativamente menor en el caso de corioamnionitis (27.6 ± 2.4 vs. 32.3 ± 3.9; $P < .0001$).

- La tasa de parto pretérmino fue del 100% en casos de corioamnionitis y del 72% (18/25) en el caso de no-corioamnionitis, (p =0.03).
- El peso al nacimiento fue 1698.4 ± 870.6 gr, y también fue estadísticamente menor en los casos de corioamnionitis comparado con el grupo sin lesiones (1069.9 ± 423.2 vs. 2169.7 ± 823.8; P < .0001).
- En total hubo 16 casos con una evaluación neurológica completamente normal al nacimiento (40%). En las otras 24 se encontró algún tipo de alteración neurológica que incluía: 4 casos (10%) de hemorragia interventricular (HIV) grado I-II, 14 casos con focos hiperecogénicos periventriculares y 2 casos de leucomalacia periventricular.
- Se hallaron diferencias estadísticamente significativas en la evaluación cerebral neonatal entre los grupos con y sin corioamnionitis (p=0.001). En total hubo 6 casos de lesión neurológica perinatal en el grupo de corioamnionitis (40%) en comparación con los 4 casos en el grupo sin corioamnionitis(16%).
- En comparación con el grupo control, la edad gestacional al parto, el peso al nacer y los niveles de glucosa en líquido amniótico fueron significativamente menores en el grupo de corioamninitis mientras que los niveles de leucocitos y de IL 6 en líquido amniótico fueron significativamente mayores. Sin embargo no se encontraron diferencias en ningunos de estos parámetros cuando se compararon en pacientes con corioamniontis y lesión neurológica perinatal, y pacientes con corioamninotis y sin lesión neurológica.

Al llevar a cabo el estudio metabolómico, se encontraron un total de 32 componentes en líquido amniótico que presentaron diferencias significativas entre las pacientes con corioamnionitis y sin corioamnionitis.

La mayoría de ellos (41%) eran metabolitos derivados de la colina, incluyendo: Lisofosfatidilcolina (60%), lisofosfatidiletanolaminas (19%), lisofosfatidilserina (3%) y fosfatidilcolina (3%). Los esfingolípidos (esfingomielinas y lactosilceramidas) contituyeron un 13%. Otros metabolitos derivados del metabolismo del piruvato, los ácidos biliares y vitamina D3 se encontraron significativamente elevados en casos de corioamnionitis.

Aquellos elementos en lo que se encontró mayores diferencias entre el grupo de la corioamnionitis y el grupo control fueron las lisofosfatidilcolinas, lisofosfatidiletanolaminas y lactosilceramidas.

Todos los elementos derivados del metabolismo lipídico especialmente lactosilceramidas mostraron las mayores diferencias entre el grupo de corioamnionitis y el grupo control.

En los casos de corioamnionitis en los que se encontraron lesiones neurológicas perinatales, se encontró que lisofosfatidilcolina (18:0) y (18:2) estaban significativamente elevadas.

Se llevó a cabo un análisis ROC con los metabolitos que mostraban una mejor correlación entre corioamnionitis aislada y corioamnionitis asociada a lesión neurológica perinatal. El análisis reveló un alto poder de discriminación para lisofosfatidiletanolamina y lactosilceramidas con un área bajo la curva > 0.95 para aquellos casos de corioamnionitis con lesión neurológica.

Para la detección de corioamnionitis con lesión neurológica, la lisofosfatidilcolina por sí sola, mostró un alto valor predictivo con un área bajo la curva de 0.99.

ESTUDIO 3

En este estudio se analizaron 20 pacientes con diagnóstico de infección intraamniótica por *Ureaplasma urealyticum.* 3 de ellas (15%) fueron casos de APP con membranas íntegras y 17 (85%) casos de rotura prematura de membranas.

En el estudio anatomopatológico de las placentas, de estos 20 casos, 8 de ellos no desarrollaron lesiones histológicas (40%). De los 12 casos con lesión, 9 de ellos (45%) presentaron corioamnionitis histológica y 3 de ellos (15%), corioamnionitis más funisitis.

Se analizaron parámetros clínicos como características maternas, edad gestacional, leucocitos y glucosa en líquido amniótico y marcadores bioquímicos incluyendo las

citoquinas: IL-1b, IL-2, IL-4, IL-6, IL 8, IL-10, IL-12, TNF-alpha, IFN- gamma, y MMP-8, buscando una correlación entre estos parámetros y el desarrollo de corioamnionitis.

Las interleuquinas 6, 8, 12, MMP8 y el recuento leucocitario estuvieron significativamente elevados en casos de corioamnionitis histológica (p = 0.007, 0.03, 0.01, 0.03, 0.03 respectivamente), mientras que la glucosa estaba disminuida (p =0.04).

Se llevó a cabo una regresión logística para encontrar parámetros para la predicción de corioamnionitis entre los casos de infección por Ureaplasma. La ecuación mostró un gran valor predictivo (R2 = 0.66, p = 0.002) incluyendo tan sólo IL6.

Si se eliminan del análisis las diferentes citoquinas analizadas, el mejor modelo predictivo incluiría los niveles de glucosa en líquido amniótico, sin embargo la capacidad predictiva disminuiría (R2 = 0.43, p =0.02).

Los valores de corte de IL 6 en líquido amniótico variarían entre 400 y 900 pg/ml dependiendo de la sensibilidad y especificidad que queramos conseguir: Para un nivel de IL 6 de 400 pg/ml tendríamos una sensibilidad para corioamnionitis en casos de infección por Ureaplasma de 100% y una especificidad de 66%. Para un punto de corte de 900 pg/ml la sensibilidad sería del 75% y la especificidad del 100%.

Como objetivo secundario del estudio buscábamos analizar el efecto de la terapia con Azitromicina en pacientes con RPM pretérmino e infección intraamniótica por Ureaplasma. Se trataron 17 de los 18 casos de RPM con un régimen de antibióticos que incluía Ampicilina, Gentamicina y Azitromicina oral en dosis de 1 gr cada 72 horas.

Cuando analizamos los resultados concluimos que de los 3 casos de funisitis identificados en toda la serie no formaban parte del grupo de pacientes que fueron tratadas con Azitromicina; dos de ellos será APP con membranas íntegras y el tercero fue un caso de RPM que se trató con Ampicilina y Gentamicina sólo.

De los 16 casos tratados con Azitromicina, la mitad desarrolló corioamnionitis histológica aislada y la otra mitad no presentaron lesiones histológicas. Ningún caso tratado con Azitromicina desarrollo funisitis

5. DISCUSIÓN GENERAL.

La infección intraamniótica es una de las principales causas de parto pretérmino; siendo responsable en un 10-20% de los casos del parto pretérmino con membranas íntegras y de hasta un 30% de las RPM pretérmino[7,11,21,22].

A lo largo de los últimos años se ha documentado la presencia de infección intraamniótica en pacientes con incompetencia cervical, placenta previa, sangrado vaginal de origen desconocido, APP y RPM [16]. Cuando esta infección intraamniótica progresa a su forma más severa se desarrolla el cuadro clínico conocido como corioamnionitis clínica que supone la finalización de la gestación. Sin embargo hoy sabemos que no todas las infecciones intraamnióticas progresan de la misma manera ni van a presentar el mismo grado de severidad. El grado de inflamación intrauterina va a depender en parte del microorganismo causante, la cantidad del inóculo, la edad gestacional en la que se inicie el proceso etc [45,46].

La ventaja del diagnóstico de una inflamación intrauterina subclínica es que representa una etapa precoz de la invasión microbiana, por lo que su detección permitiría ofrecer un pronóstico más certero y un mejor manejo de la gestación e hipotéticamente iniciar un tratamiento antibiótico dirigido en una etapa temprana precoz. Es por ello que la investigación actual se centra en la búsqueda de marcadores que no sólo identifiquen la presencia de una inflamación intraamniótica si no que sean capaces de diferenciar entre los diferentes grados del proceso inflamatorio para un mejor manejo de estas gestaciones.

Hasta el momento, la IL6 es el marcador de inflamación intraamniótica más estudiado y más sensible según la evidencia científica actual [95-99].Sin embargo el principal problema de IL6 continua siendo su alta tasa de falsos positivos (17-34%) [99] que limita su aplicabilidad clínica ya que puede derivar en la finalización de gestaciones a una edad gestacional muy temprana. Del mismo modo, no existen hasta la fecha unos valores de IL6 u otro biomarcador en líquido amniótico que nos permita diferenciar entre diferentes grados de inflamación intrauterina que podría ser especialmente

útiles a la hora de planificar el manejo de la gestación en aquellas infecciones causadas por microorganismos de baja virulencia que normalmente se asocian con bajos grados de inflamación y baja morbilidad neonatal como *Ureaplasma urealyticum.*

Los resultados del Estudio 1 muestran que, mientras que el 83% de las pacientes con sospecha de infección intraamniótica desarrollaron una corioamnionitis histológica, tan sólo el33% de ellas evolucionaron a funsitis, la manifestación clínica del SRIF.

De estas 10 moléculas, tan sólo 3, IL 6, IL 8 e IL 12 presentaron diferencias significativas en las tres comparaciones realizadas: control vs. corioamnionitis aislada, corioamnionitis aislada vs. corioamnionitis + funisitis y control vs corioamnionitis + funisitis. De esto, se podría deducir que cada una de estas tres moléculas por sí sola podría diferenciar por un lado, la presencia de inflamación y otro, el grado de inflamaciónintrauterina.

La relación entre IL 6 e IL 8 con el SRIF ha sido bien establecida hasta el momento [78,109-112]. Sin embargo la relación con IL 12 es más desconocida llegando incluso algunos trabajos a rechazar el papel de esta IL en el diagnóstico de SRIF [112-114].

El hecho de que estos estudios presentaran una muestra de estudio pequeña, que relacionaran el nivel de IL con resultados del cultivo microbiológico en vez del estudio histológico de la placenta y que estudiaran el nivel de IL en sangre fetal en vez de en líquido amniótico, podrían explicar las diferencias con nuestros resultados, que señalan a IL 12 como una de las moléculas más predictivas entre las estudiadas para el diagnóstico de inflamación histológica.

Durante el estudio se llevó a cabo una regresión logística para encontrar una combinación de IL capaz de predecir la presencia de funisitis en pacientes con infección intraamniótica. La fórmula resultante presentaba un gran valor predictivo e incluía 4 de las 10 citoquinas estudiadas: IL-4, IL-8, IL-10, y IL-12, que sorprendentemente no se encuentran entre las moléculas más estudiadas ni relacionadas con el SRIF hasta la fecha. Parece que la combinación de estas 4 citoquinas inflamatorias pueden predecir prácticamente la totalidad de los casos de

funisitis en pacientes en riesgo con más precisión que cualquier citoquina aislada, incluyendo IL6.

Según los resultados arrojados por el presente estudio, parece que, a pesar de que las moléculas clásicamente relacionadas con el SRIF como IL6 y MMP 8 presentan un elevada sensibilidad para el diagnóstico de inflamación histológica, no presentan una alta capacidad predictiva a la hora de diferenciar entre la presencia de corioamnionitis y corioamnionitis + funisitis.

Este modelo de predicción matemático incluye en la fórmula dos moléculas pro inflamatorias (IL 8 e IL 12) y dos moléculas antiinflamatorias (IL 4 e IL 10). Teniendo en cuenta esto, podríamos especular que un incremento en los niveles de citoquinas antiinflamatorias tendría un papel compensatorio en el proceso de inflamación intrauterina, y que por lo tanto, un pérdida en el equilibrio entre moléculas pro y antiinflamatorias podría favorecer el desarrollo de un SRIF.

En un futuro, puede que sea la combinación de varias marcadores en vez de valores de citoquinas aisladas, las que nos lleve más cerca de poder identificar los diferentes grados de afectación intrauterina en el complejo infección/inflamación intraamniótica.

En el ESTUDIO 2 se buscaba identificar un perfil metabolómico capaz de identificar pacientes con corioamnionitis subclínica y aquellas que presentaban un alto riesgo de desarrollan lesión neurológica perinatal.

Hasta el momento existen pocos trabajos que estudien la metabolómica en líquido amniótico [108] y ninguno de ellos fue diseñado para el estudio de la fisiopatología de la inflamación intraamniótica y morbilidad neonatal.

En este estudio se mostró que el grupo de los esfingolípidos, entre ellos esfingomielina y lactosilceramida, mostraron diferencias estadísticamente significativas entre el grupo de corioamnionitis histológica y el grupo control.

Los esfingolípidos son una clase de lípidos implicados en la protección de la superficie celular contra factores externos formando una capa superficial químicamente resistente a los diferentes elementos dañinos del exterior. Dentro de ellos, las

lactosilceramidas mostraron las mayores diferencias entre todos los metabolitos analizados, presentándose en concentraciones 3000 veces mayores en el grupo de corioamnionitis comparado con el grupo control.

Esto puede llevar a la suposición de que estas moléculas pueden llegar a ser consideradas como biomarcadores muy útiles en el diagnóstico de este proceso.

Las lactosilceramidas son conocidos agentes apoptóticos en las células del epitelio amniótico que produce una liberación de prostaglandinas junto con la inducción de la apoptosis de las células epiteliales amnióticas, de tal manera que el metabolismo de las prostaglandinas amnióticas está ligada con la apoptosis en las células epiteliales del amnion y esto puede llevar a la rotura de membranas. En este estudio se evidenció niveles muy incrementados de lactosilceramidas en casos de corioamnionitis y fuertemente correlacionados con los niveles de IL6 y de glucosa en líquido amniótico.

Las lactosilceramidas también se consideran un mensajero secundario en los procesos neuroinflamatorios [115]. Es un importante elemento de señalización para la inducción de mediadores proinflamatorios y astrogliosis [116]. Estas moléculas también promueven la producción de óxido nítrico estimulado por las citoquinas inflamatorias especialmente en astrocitos y en el tejido cerebral. Sin embargo en este estudio no se pudo encontrar diferencias significativas en los niveles de lactosilceramidas en pacientes con corioamnionitis y lesión neurológica perinatal. Hay que tener en cuenta sin embargo, que en este estudio la mayor parte de las lesiones neurológicas perinatales fueron casos de hemorragia interventricular. Las lesiones que clásicamente se han relacionado con un proceso inflamatorio a nivel cerebral son la leucomalacia periventricular y las secuelas a largo plazo. Por lo tanto podemos concluir que la lactosilceramida está muy incrementada en casos de corioamnionitis y puede ser propuesto como candidato a marcador diagnóstico de este proceso, sin embargo, no parece ser predictivo de hemorragiainterventricular.

Otro grupo de metabolitos que mostraron diferencias significativas en mujeres con y sin corioamnionitis fueron los glicerofosfolípidos, incluyendo Lisofosfatidilcolina, lisofosfatidiletanolaminas, lisofosfatidilserina y fosfatidilcolina. Todas estas moléculas

son, además de componentes de la membrana extracelular, importantes mensajeros secundarios con un amplio espectro de efectos biológicos [117].

Se han reportado niveles elevados de lisofosfatilcolina en tejido cerebral tras situaciones de isquemia cerebral, epilepsia e inflamación [118] además de ser un conocido agente promotor de la desmielinización en el sistema nervioso central [119]. Estos hallazgos se corresponden con nuestros resultados, en los que se observaban niveles significativamente elevados de lisofosfatilcolina en casos de corioamnionitis con complicaciones neurológicas postnatales. Tanto es así que, al llevar cabo el análisis ROC para la predicción de corioamnionitis con lesión neurológica perinatal, la lisofosfatidilcolina por sí sola, mostró un alto valor predictivo, con un área bajo la curva de 0.99.

La lisofatidiletanolamina presentó también resultados interesantes, siendo las moléculas que más se correlacionaron con los niveles de IL6, glucosa y leucocitos en líquido amniótico y siendo junto con la lisofosfatidilcolina y la lactosilceramida las moléculas más relacionadas con los casos de corioamnionitis subclínica.

En casos de corioamnionitis, el grupo de ácidos biliares presentaron niveles significativamente elevados en los casos de lesión neurológica perinatal. Particularmente los ácidos sulfocólicos y trioxocolenoico presentaron niveles muy elevados en el líquido amniótico en aquellos fetos que posteriormente desarrollaron una hemorragia interventricular. Este tipo de componentes se han relacionado con fenómenos trombóticos de la arteria cerebral media y daño endotelial severo [120] además de haber demostrado alterar la barrera hematoencefálica y permitir la extravasación de sangre desde los vasos hacia el tejido cerebral [121]. Sin embargo, se necesitan más estudios que permitan definir bien la relación entre el incremento de ácidos biliares y la hemorragia interventricular en el contexto de una inflamación intrauterina.

En el ESTUDIO 3 pretendíamos estudiar más a fondo la infección intraamniótica por *Ureaplasma urealyticum.* En primer lugar porque Ureaplasma es el microorganismo más comúnmente aislado en casos de infección intraamniótica, aproximadamente en

el 35% de los casos [42,43] y en segundo lugar, porque actualmente existe la creencia de que este microorganismo de baja virulencia puede llegar ,en algunos casos ,a colonizar el especio coriodecidual sin llegar a progresar a niveles más severos de inflamación. De tal manera que, según algunos trabajos [34,35] la infección intraamniótica por este microorganismo causa parto pretérmino en menos del 25% de los casos mientras que la mayoría de las gestaciones evolucionan normalmente hasta el término.

Nuestros resultados fueron acordes a los datos publicados anteriormente. De las 20 pacientes en las que se objetivó infección amniótica por *Ureaplasma urealyticum* menos de la mitad de los casos desarrollaron corioamnionitis histológica, el 45%, mientras que tan sólo 3 de los casos (15%) llego a desarrollarfunisitis.

Existen varios artículos que estudian las infección por Ureaplasma y su relación con citoquinas inflamatorias [32,36,122]. Sin embargo, este el primer trabajo que estudia un amplio panel de moleculas inflamatorias y su relación con inflamación histológica causada por Ureaplasma.

De todas las citoquinas estudiadas, tan sólo IL6, IL 8, IL12 y MMP8 presentaron diferencias significativas entre los grupos con y sin inflamación histológica. Estos resultados son acordes con los arrojados por el ESTUDIO 1 en el que se comprobó que las moléculas IL6, 8 y 12 eran capaces por sí solas, de diferenciar entre los diferentes estados de inflamación histológica en el contexto de una infección intraamniótica.

De todas estas moléculas, en casos de infección por Ureasplama, IL 6 fue la que presentó más capacidad predictiva de corioamnionitis. En nuestra muestra, unos valores superiores a 400 pg/ml detectarían un 100% de los casos de corioamnionitis, mientras que unos valores superiores a 900 pg/ml aumentaría la especificidad hasta el 100% con una tasa de detección del 75%.

El nuevo objetivo en la investigación de la infección intraamniótica es el desarrollo de tratamientos dirigidos a erradicar la infección en estadíos precoces y evitar así la cascada inflamatoria que puede culminar en parto pretérmino y morbilidad materna y neonatal.

Actualmente es generalizado el uso de tratamiento antibiótico en casos de RPM pretérmino, siendo la Eritromicina el antibiótico más comúnmente aceptado en el tratamiento de la infección por Ureaplasma. Sin embargo, la Eritromicina ha demostrado tener un limitado paso placentario y ha fallado a la hora de demostrar la erradicación del microorganismo o mejorar los resultados neonatales. En los últimos años han surgido ciertos trabajos [46,123,124] que han reportado en modelos animales, la erradicación de Ureaplasma intraamniótica con la administración intravenosa materna de Azitromicina.

Nosotros quisimos evaluar nuestros resultados tras la incorporación de Azitromicina oral a la pauta antibiótica en el tratamiento de las RPM pretémino. Se trataron 16 pacientes con RPM con régimen de Ampicilina 1 g IV/6h + Gentamicina 240mg/24h + Azitromicina 1g oral/72 h. De estos casos, la mitad de ellos no presentó ningún tipo de inflamación histológica en el estudio placentario, y ninguno de ellos desarrolló funisitis.

Estos resultados han de considerarse con precaución dado que por un lado, la muestra es demasiado pequeña para sacar conclusiones y por otra nuestra posología fue diferente a la utilizada en los estudios anteriormente mencionados. Sin embargo, con la evidencia actual, se abre una puerta a la investigación de nuevas terapias encaminadas a la erradicación de la infección intraamniótica y en un futuro puede que sea la administración de Azitromicina la clave para el tratamiento y erradicación del Ureaplasma

6. CONCLUSIONES.

CONCLUSIONES DEL ESTUDIO 1

- El proceso de inflamación intraamniótica es un proceso variable con diferentes grados de severidad. Entre las pacientes con riesgo de infección intraamniótica, esto es, rotura prematura de membranas y amenaza de parto pretérmino con factores de mal pronóstico, se desarrolló una inflamación histológica en un 37.5% de las pacientes, sin embargo, tan sólo una pequeña proporción de casos, un 15%, evolucionó a funisitis, la expresión histológica del SRIF.
- La causa subyacente de estos procesos de inflamación intrauterina es la infección intraamniótica en la mayoría de los casos, un 80% de los casos en nuestra serie.
- La combinación de IL 12, 10, 4 y 8 en líquido amniótico tiene un alto valor predictivo para la detección de funisitis en pacientes con riesgo de infección intraamniótica.
- En un futuro, puede que sea la combinación de citoquinas inflamatorias, y no la determinación de una IL aislada la que nos lleve a una identificación de los diferentes grados de inflamación histológica en el contexto de una infección intraamniótica.

CONCLUSIONES DEL ESTUDIO 2

- La metabolómica es una herramienta sensible para la identificación de un perfil de metabolitos en líquido amniótico relacionado con la infección intraamniótica y podría a ser crucial a la hora de identificar biomarcadores diagnósticos y terapéuticos de coriomanionitis y lesión neurológica perinatal.
- La acumulación de ciertas ceramidas en líquido amniótico relacionadas con procesos apoptóticos en las membranas amnióticas son marcadores sensibles de corioamnionitis. El grupo de los esfingolípidos , especialmente esfingomielina y lactosilceramidas fueron los metabolitos que diferenciaban aquellas pacientes con corioamnionitis
- El aumento de algunos ácidos biliares en un ambiente proinflamatorio podría

ser importante en la predicción y la detección de aquellos neonatos con alto riesgo de desarrollar hemorragia interventricular.

- Lisofosfatidilcolina, el ácido sulfocólico y especialmente el ácido trioxocolenoico podrían ser considerados como posibles marcadores diagnósticos en la identificación de neonatos con daño neurológico perinatal asociado a corioamnionitis materna.
- La metabólomica es una nueva técnica que aunque prometedora, aún no ha sido ampliamente estudiada en el contexto de la corioamnionitis subclínica y sus consecuencias perinatales. Se necesitan más estudios para poder establecer el verdadero potencial de esta técnica en el complejo infección/inflamación intraamniótica.

CONCLUSIONES DEL ESTUDIO 3

- La infección intraamniótica por Ureaplasma en pacientes con APP o RPM pretérmino no está asociada en todos los casos con una inflamación histológica. Aproximadamente el 40% no desarrollará ningún tipo de lesión.
- A la hora de identificar aquellos casos que desarrollarán corioamnionitis, IL 6, IL 8, IL 12 y MMP 8 podrían considerarse marcadores diagnósticos de lesión.
- Valores de IL 6 en líquido amniótico por encima de 400 pg/ml detectó el 100% de los casos de corioamnionitis con una especificidad del 66%. Si elevamos el punto de corte a 900 pg/ml, aumentaremos la especificidad hasta un 100% con una sensibilidad del 75%.
- La Azitromicina podría ser una opción terapeútica eficaz para la erradicación de la infección intraamniótica por Ureaplasma. En nuestra serie, ninguna de las pacientes tratadas con Azitromicina presentaron funisitis y la mitad no desarrollaron lesión histológica alguna. Sin embargo se necesitan más estudios para determinar su verdadera efectividad y establecer la mejor pauta de tratamiento.

7. BIBLIOGRAFÍA

1. Spong, C.Y; Preterm birth: an enigma and a priority. Obstet Gynecol, 2009. 113(4): p. 770-1.
2. Blencowe H et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet. 2012;379:2162-2172.
3. Chang HH et al. Preventing preterm births: analysis of trends and potential reductions with interventions in 39 countries with very high human development index. Lancet. 2013;381:223-234.
4. Protocolos SEGO: Rotura Prematura de Membranas. Disponible en http://www.prosego.com
5. Protocolos SEGO: Amenaza de parto pretérmino. Disponible en http://www.prosego.com.
6. Goldenberg, R.L. et al.; Epidemiology and causes of preterm birth. Lancet, 2008. 371 (9606): p. 75-84.
7. Romero, R. et al; The preterm labor syndrome. Ann N Y Acad Sci, 1994. 734: p. 414-29
8. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science. 2014;345(6198):760–5.
9. Romero R, Mazor M. Infection and preterm labor. Clin Obstet Gynecol. 1988; 31(3):553–84.
10. Romero R, Mazor M, Munoz H, Gomez R, Galasso M, Sherer DM. The preterm labor syndrome. Ann N Y Acad Sci. 1994;734:414–29.
11. Goncalves LF, Chaiworapongsa T, Romero R. Intrauterine infection and prematurity. Ment Retard Dev Disabil Res Rev. 2002;8(1):3–13.
12. Romero R, et al. The preterm parturition syndrome. BJOG. 2006; 113(Suppl 3):17–42.
13. Benirschke K. Routes and types of infection in the fetus and the newborn. AMA J Dis Child. 1960;99:714–21.
14. Hein M, Helmig RB, Schonheyder HC, Ganz T, Uldbjerg N. An in vitro study of antibacterial properties of the cervical mucus plug in pregnancy. Am J Obstet Gynecol. 2001;185(3):586–92.
15. Hein M, Valore EV, Helmig RB, Uldbjerg N, Ganz T. Antimicrobial factors in the cervical mucus plug. Am J Obstet Gynecol. 2002;187(1):137–44.
16. Kim CJ, Romero R, Chaemsaithong P, Chaiyasit N, Yoon BH, Kim YM. Acute Chorioamnionitis and Funisitis: Definition, Pathologic Features, and Clinical Significance. Am J Obstet Gynecol. 2015;213(4):S29-52.
17. Romero R, Espinoza J, Chaiworapongsa T, Kalache K. Infection and prematurity and the role of preventive strategies. Semin Neonatol 2002; 7(4):259-74.
18. Galask RP, Varner MW, Petzold CR, Wilbur SL. Bacterial attachment to the chorioamniotic membranes. Am J Obstet Gynecol. 1984;148(7):915–28.
19. Gravett MG, Hummel D, Eschenbach DA, Holmes KK. Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. Obstet Gynecol. 1986;67(2):229–37.
20. Romero R, Gomez R, Chaiworapongsa T, Conoscenti G, Kim JC, Kim YM. The role of infection in preterm labour and delivery. Paediatr Perinat Epidemiol. 2001; 15 (2):41–56.
21. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. Semin Reprod Med. 2007; 25(1):21–39.

22. Goldenberg, RL. Hauth J.C, Andrews W.W. Intrauterine infection and preterm delivery. N Engl J Med, 2000. 342(20): p. 1500-7.
23. Lau, J. et al; Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than chorioamnionitis displaying a maternal inflammatory response only. Am J Obstet Gynecol, 2005. 193 (3 Pt 1): p. 708-13.
24. Carroll, S.G et al. Maternal assestment in the prediction of intrauterine infection in preterm labor amniorrhexis. Fetal Diagn Ther, 1995. 10(5): p. 290-6
25. Gibbs, RS et al; Quantitative bacteriology of amniotic fluid from woman with clinical intraamniotic infection at term. J Infect Dis, 1982. 145(1): p. 1-8.
26. Romero R; et al. Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in woman with preterm labor and intact membranes. Am J Obstet Gynecol, 1989. 161(3): p. 817-24.
27. Offenbacher S, et al. Maternal periodontitis and prematurity. Part I: Obstetric outcome of prematurity and growth restriction. Ann Periodontol. 2001; 6(1):164–74.
28. Oh KJ, Lee SE, Jung H, Kim G, Romero R, Yoon BH. Detection of ureaplasmas by the polymerase chain reaction in the amniotic fluid of patients with cervical insufficiency. J Perinat Med. 2010; 38(3):261–8.
29. Gravett MG, Eschenbach DA. Possible role of Ureaplasma urealyticum in preterm premature rupture of the fetal membranes. Pediatr Infect Dis. 1986; 5(6 Suppl):S253–7.
30. Yoon BH et al. Microbial invasion of the amniotic cavity with Ureaplasma urealyticum is associated with a robust host response in fetal, amniotic, and maternal compartments. Am J Obstet Gynecol. 1998;179(5):1254–60.
31. Yoon BH al. Clinical implications of detection of Ureaplasma urealyticum in the amniotic cavity with the polymerase chain reaction. Am J Obstet Gynecol. 2000; 183(5):1130–7.
32. Yoon BH et al. The clinical significance of detecting Ureaplasma urealyticum by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. Am J Obstet Gynecol. 2003;189(4):919–24.
33. Kim M, Kim G, Romero R, Shim SS, Kim EC, Yoon BH. Biovar diversity of Ureaplasma urealyticum in amniotic fluid: distribution, intrauterine inflammatory response and pregnancy outcomes. J Perinat Med. 2003; 31(2):146–52.
34. Gerber S, Vial Y, Hohlfeld P, Witkin SS. Detection of Ureaplasma urealyticum in second-trimester amniotic fluid by polymerase chain reaction correlates with subsequent preterm labor and delivery. J Infect Dis. 2003;187(3):518–21.
35. Perni et al. Mycoplasma hominis and Ureaplasma urealyticum in midtrimester amniotic fluid: association with amniotic fluid cytokine levels and pregnancy outcome. Am J Obstet Gynecol. 2004;191(4):1382–6.
36. Jacobsson B, Aaltonen R, Rantakokko-Jalava K, Morken NH, Alanen A. Quantification of Ureaplasma urealyticum DNA in the amniotic fluid from patients in PTL and pPROM and its relation to inflammatory cytokine levels. Acta Obstet Gynecol Scand. 2009;88(1):63–70.

37. Romero R, et al. Clinical chorioamnionitis at term I: microbiology of the amniotic cavity using cultivation and molecular techniques. J Perinat Med. 2015; 43(1):19–36.
38. Romero R, et al. A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. Am J Reprod Immunol. 2014; 71(4):330–58.
39. Romero R, Reece EA, Duff GW, Coultrip L, Hobbins JC. Prenatal diagnosis of Candida albicans chorioamnionitis. Am J Perinatol. 1985; 2(2):121–2.
40. Romero R, et al. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2014:1–16.
41. Digiulio DB, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. PLoS One. 2008; 3(8):e3056.
42. Revello R, Alcaide MJ, Dudzik D et al. Differential amniotic fluid cytokine profile in women with chorioamnionitis with and without funisitis. J Matern Fetal Neonatal Med. 2016; 29:2161-5.
43. Goldenberg RL, al. The Alabama Preterm Birth Study: umbilical cord blood Ureaplasma urealyticum and Mycoplasma hominis cultures in very preterm newborn infants. Am J Obstet Gynecol. 2008; 198:43.
44. Aaltonen R, Heikkinen J, Vahlberg T et al. Local inflammatory response in choriodecidua induced by Ureaplasma urealyticum. BJOG. 2007; 114:1432-5.
45. Kacerovsky M, Pliskova L, Menon R, et al. Microbial load of umbilical cord blood Ureaplasma species and Mycoplasma hominis in preterm prelabor rupture of membranes. J Matern Fetal Neonatal Med. 2014; 27:1627-32.
46. Grigsby PL, Novy MJ, Sadowsky DW, et al. Maternal azithromycin therapy for Ureaplasma intraamniotic infection delays preterm delivery and reduces fetal lung injury in a primate model. Am J Obstet Gynecol. 2012; 207:475.
47. Martinez de Tejada B, Coll O, De Flores M, Hiller SL, Landers DV. Prevalencia de la vaginosis bacteriana en una población obstétrica de Barcelona. Med Clin (Barc). 199821;110(6):201-4.
48. Guise JM, Mahon S, Aickin M, Helfand M. Screening for Bacterial Vaginosis in Pregnancy. Disponible en :http://www.ncbi.nlm.hih.gov/books/NBK42659/.
49. Pararas MV,Skevaki CL, Kafetzis DA. Preterm bith due to maternal infection: Causative pathogens and modes of prevention. Eur J Clin Microbiol Infect Dis, 2006. 25(9): p.562-9.
50. Klein LL, Gibbs RS. Use of microbial cultures and antibiotics in the prevention of infection-associated preterm birth. Am J Obstet Gynecol, 2004. 190(6):p. 1493-502.
51. Romero R, et al. Meta-analysis of the relationship between asymptomatic bacteriuria and preterm delivery/low birth weight. Obstet Gynecol, 1989. 73(4): p. 576 -82.
52. Garite TJ, Freeman RK, Linzey E, Braley P. The use of amniocentesiss in patients with premature rupture of membranes. Obstet Gynecol 1979; 54:226-230-
53. Garite TJ. Premature ruptue of membranes: the eniga for the obstetrician. Am J Obstet Gynecol 1985; 151:1001-1005.

54. Leigh J, Garite TJ: Amniocnetesis in the management of preterm labor. Obstet Gynecol 1986;67:500-506.
55. Midtrimester amniocentesis for prenatal diagnosis. Safety and accuracy. Jama, 1976. 236(13): p.147-6.
56. Galle PC, Meis PJ. Complications of amniocentesis: a review. J Reprod Med, 1982. 27(3): p.149-55.
57. Le Bouar G, Lassel L, Poulain P. Markers of infection and inflammation in the amniotic fluid: therapeutic contribution of amniocentesis. J Gynecol Obstet Biol Reprod (Paris). 2002; 31(7):5S52-6.
58. Espinoza J et al. The prevalence and clinical significance of amniotic fluid 'sludge' in patients with preterm labor and intact membranes. Ultrasound Obstet Gynecol. 2005;25(4):346–52.
59. Bujold E et al. Intra-amniotic sludge, short cervix, and risk of preterm delivery. J Obstet Gynaecol Can. 2006;28(3):198–202.
60. Romero R et al. What is amniotic fluid 'sludge'? Ultrasound Obstet Gynecol. 2007; 30(5):793–8.
61. Kusanovic JP et al. Clinical significance of the presence of amniotic fluid 'sludge' in asymptomatic patients at high risk for spontaneous preterm delivery. Ultrasound Obstet Gynecol. 2007;30(5):706–14.
62. Romero R et al. Detection of a microbial biofilm in intraamniotic infection. Am J Obstet Gynecol. 2008; 198(1):135, e1–5. [PubMed: 18166328]
63. Hatanaka AR, Mattar R, Kawanami TE, Franca MS, Rolo LC, Nomura RM, et al. Amniotic fluid "sludge" is an independent risk factor for preterm delivery. J Matern Fetal Neonatal Med.2014:1–6.
64. Fuchs F, Boucoiran I, Picard A, Dube J, Wavrant S, Bujold E, et al. Impact of amniotic fluid "sludge" on the risk of preterm delivery. J Matern Fetal Neonatal Med. 2014:1–5.
65. Boyer A, Cameron L, Munoz-Maldonado Y, Bronsteen R, Comstock CH, Lee W, et al. Clinical significance of amniotic fluid sludge in twin pregnancies with a short cervical length. Am J Obstet Gynecol. 2014; 211(5):506, e1–9.
66. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284(5418):1318–22.
67. Lee JH, Romero R, Kim SM, Chaemsaithong P, Park CW, Park JS, et al. A new antimicrobial combination prolongs the latency period, reduces acute histologic chorioamnionitis, and funisitis, and improves neonatal outcomes in preterm PROM. J Matern Fetal Neonatal Med. 2016 Sep;29(17):2727-37.
68. Hassan S et al. A sonographic short cervix as the only clinical manifestation of intra-amniotic infection. J Perinat Med. 2006;34(1):13–9.
69. Gomez R et al. Antibiotic administration to patients with preterm premature rupture of membranes does not eradicate intra-amniotic infection. J Matern Fetal Neonatal Med.2007;20(2):167-73.
70. Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, Vogler C. Amniotic infection syndrome: nosology and reproducibility of placental reaction patterns. Pediatr Dev Pathol. 2003;6(5):435–48.
71. Benirschke, K.; Burton, GJ.; Baergen, RN. Pathology of the Human Placenta. Sixth ed.. Springer; Berlin Heidelberg: 2012. Infectious Diseases.; p. 557-656.

72. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001; 1(2):135–45.
73. Kim CJ, et al. Umbilical arteritis and phlebitis mark different stages of the fetal inflammatory response. Am J Obstet Gynecol. 2001; 185(2):496–500.
74. Redline RW. Inflammatory response in acute chorioamnionitis. Sem Fetal Neonatal Med.2012;17(1):20-5.
75. Lahra MM, Jeffery HE. A fetal response to chorioamnionitis is associated with early survival after preterm birth. Am J Obstet Gynecol 2004; 190:147.
76. Yoon BH et al. Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Obstet Gynecol. 2001;185(5):1130
77. Shim SS, et al. Clinical significance of intra-amniotic inflammation in patients with preterm rupture of membranes. Am J Obstet Gynecol, 2004. 191(4):p. 1339-45.
78. Gomez R et al; The fetal inflammatory response syndrome. Am J Obstet Gynecol, 1998. 179(1): p. 194-202.
79. Wharton KN, et al. Severe umbilical cord inflammation-a predictor pf periventricular leukomalacia in very low birth weight infants. Early Hum Dev, 2004. 77(1-2): p. 77-87.
80. Naeye RL. Functionally important disorders of the placenta, umbilical cord, and fetal membranes. Hum Pathol, 1987. 18(7): p. 680-91.
81. Lee SD et al. Chorionic plate vessel as an origin of amniotic fluid neutrophils. Pathol Int, 2004. 54(7): p.516-22.
82. Yoon BH et al. Amniotic fluid inflammatory citokines (interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. Am J Obstet Gynecol.1997;177(1):19-26.
83. Yoon BH, Park CW, Chaiworapongsa T. Intrauterine infection and the development of cerebral palsy.BJOG.2003;110(20):124-7.
84. Romero R et al. Fetal cardiac disfunction in preterm premature rupture of membranes. J Matern Fetal Neonatal Med. 2004;16(3): 146-57.
85. Wu YW, Colford JM, Chorioamnionitis as a risk factor for cerebral palsy: A meta-analysis. Jama, 2000. 284(11):p1417-24.
86. Di Naro et al. Myocardial dysfunction in fetuses exposed to intraamniotic infection: new insights from tissue Doppler and strain imaging. Am J Obstet Gynecol. 2010;203(5):459.
87. Letti Muller AL et al. Tei index to assess fetal cardiac performance in fetuses at risk for fetal inflammatory reponse syndrome. Ultrasouns Obstet Gynecol. 2010;36(1):26-31.
88. Lee SY, Leung CW. Histological chorioamnionitis- implication for bacterialcolonization, laboratory markers of infection, and early onset sepsis in very low birth weight neonates. J Matern Fetal Neonatal Med.2012;25(4):364-8.
89. Romero R et al. Infection and labor. III. Interleukin-1: a signal for the onset of parturition. Am J Obstet Gynecol. 1989; 160(5 Pt 1):1117–23. [PubMed: 2786341]
90. Romero R et al. Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition. Am J Reprod Immunol. 1992; 27(3-4):117–23.

91. Figueroa R, Garry D, Elimian A, Patel K, Sehgal PB, Tejani N. Evaluation of amniotic fluid cytokines in preterm labor and intact membranes. J Matern Fetal Neonatal Med. 2005;18(4):241–7.
92. Cox SM, Casey ML, Macdonald PC. Accumulation of interleukin-1beta and interleukin-6 in amniotic fluid: a sequela of labour at term and preterm. Hum Reprod Update. 1997;3(5):517–27.
93. Romero R, Avila C, Santhanam U, Sehgal PB. Amniotic fluid interleukin 6 in preterm labor. Association with infection. J Clin Invest. 1990;85(5):1392–400.
94. Romero R, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Reprod Immunol. 2014;72(5):458–74.
95. Hillier SL, et al. The realationship of amniotic fluid cytokines and preterm delivery,amniotic fluid infection, histologic chorioamnionitis, and chorioamnionitis infection. Obstet Gynecol, 1993.(1(6): p.941-8.
96. Coultrip LL, et al. The value of amniotic fluid interleukin-6 determination in patients with preterm labor and intact membranes in the detection of microbial invasion of the amniotic cavity. Am J Obstet Gynecol, 1994. 171(4): p901-11.
97. El-Bastawissi AY, et al. Amniotic fluid interleukin-6 and preterm delivery: a review. Obstet Gynecol, 2000. 95(6Pt2): p.1056-64.
98. Jacobsson B, et al. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women with preterm prelabor rupture of membranes. Acta Obstet Gynecol Scand, 2003. 82(2):p.423-31
99. Romero R, et al. The diagnostic and prognostic value of amniotic fluid white blood cell count, glucose, interleukin-6, and gram stain in patients with preterm labor and intact membranes. Am J Obstet Gynecol, 1993. 169(4): p.805-16.
100. Sampson SE, Theve RP, Blatman RN, Shipp TD, Bianchi DW, Ward BE, et al. Fetal origin of amniotic fluid polymorphonuclear leukocytes. Am J Obstet Gynecol. 1997; 176:77–81
101. Angus SR, Segel SY, Hsu CD, Locksmith GJ, Clark P, Sammel MD, et al. Amniotic fluid matrix metalloproteinase-8 indicates intra-amniotic infection. Am J Obstet Gynecol. 2001;185:1232–1238.
102. Maymon E, Romero R, Chaiworapongsa T, Berman S, Conoscenti G, Gomez R, et al. Amniotic fluid matrix metalloproteinase-8 in preterm labor with intact membranes. Am J Obstet Gynecol. 2001; 185:1149–1155. [PubMed: 11717649]
103. Maymon E, Romero R, Chaiworapongsa T, Kim JC, Berman S, Gomez R, et al. Value of amniotic fluid neutrophil collagenase concentrations in preterm premature rupture of membranes. Am J Obstet Gynecol. 2001; 185:1143–1148. [PubMed: 11717648]
104. Maymon E, Romero R, Pacora P, Gomez R, Athayde N, Edwin S, et al. Human neutrophil collagenase (matrix metalloproteinase 8) in parturition, premature rupture of the membranes, and intrauterine infection. Am J Obstet Gynecol. 2000; 183:94–99.
105. Nien JK, et al. A rapid MMP-8 bedside test for the detection of intraamniotic inflammation identifies patients at risk for inminent preterm delivery. Am J Obstet Gynecol, 2006. 195(4): p.1025-30.

106. Yoon BH, Romero R, Yang SH, et al. Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. Am J Obstet Gynecol.1996;174(5):1433-1440.
107. Bugatto F, Fernández-Deudero A, Bailén A, Fernández-Macías R, Hervías-Vivancos, B, Bartha JL. Second-trimester amniotic fluid proinflammatory cytokine levels in normal and overweight women. Obstet Gynecol.2010;115(1):127-133.
108. Kamath-Rayne BD, Smith HC, Muglia LJ, Morrow AL. Amniotic fluid: the use of high-dimensional biology to understand fetal well-being. Reprod Sci. 2014;21(1):6-19.
109. Brown AS, et al. Elevated maternal interleukin-8 levels and risk of schizophrenia in adult offspring. Am J Psychiatry2004;161:889–95.
110. Kacerovsky M, et al. The fetal inflammatory response in subgroups of women with preterm prelabor rupture of the membranes. J Matern Fetal Neonatal Med 2013;26:795–801.
111. Satar M et al. Cord blood cytokine levels in neonates born to mothers with prolonged premature rupture of membranes and its relationship with morbidity and mortality. Eur Cytokine Netw2008;19:37–41.
112. Cift T, Uludag S, Aydin Y, Benian A. Effects of amniotic and maternal CD-146, TGF-b1, IL-12, IL-18 and IFN-g, on adverse pregnancy outcome. J Matern Fetal Neonatal Med 2013;26:21–5.
113. Ekelund CK, Vogel I, Skogstrand K, et al. Interleukin-18 and interleukin-12 in maternal serum and spontaneous preterm delivery. J Reprod Immunol 2008;77:179–85.
114. Edwards RK, Clark P, Locksmith Gregory J, Duff P. Performance characteristics of putative tests for subclinical chorioamnionitis. Infect Dis Obstet Gynecol 2001;9:209–14.
115. Won JS, Singh AK, Singh I. Lactosylceramide: a lipid second messenger in neuroinflammatory disease. J Neurochem. 2007;103 Suppl 1:180-191.
116. Pannu R, Singh AK, Singh I. A novel role of lactosylceramide in the regulation of tumor necrosis factor alpha-mediated proliferation of rat primary astrocytes. Implications for astrogliosis following neurotrauma. J Biol Chem. 2005;280(14):13742-13751.
117. Dong J, Cai X, Zhao L, et al. Lysophosphatidylcholine profiling of plasma: discrimination of isomers and discovery of lung cancer biomarkers. Metabolomics. 2010;6(4):478-488.
118. Stock J. The emerging role of lipidomics. Atherosclerosis. 2012;221(1):38-40.
119. Sevastou I, Kaffe E, Mouratis MA, Aidinis V. Lysoglycerophospholipids in chronic inflammatory disorders: the PLA(2)/LPC and ATX/LPA axes. Biochim Biophys Acta. 2013;1831(1):42-60.
120. Kang EJ, Major S, Jorks D, et al. Blood-brain barrier opening to large molecules does not imply blood-brain barrier opening to small ions. Neurobiol Dis. 2013;52:204-218.
121. Jorks D, Milakara D, Alam M, et al. A novel algorithm for the assessment of bloodbrain barrier permeability suggests that brain topical application of endothelin-1 does not cause early opening of the barrier in rats. Cardiovasc Psychiatry Neurol.2011;2011:169580.

122. Yoneda N, Yoneda S, Niimi H. Polymicrobial Amniotic Fluid Infection with Mycoplasma/Ureaplasma and Other Bacteria Induces Severe Intra-Amniotic Inflammation Associated with Poor Perinatal Prognosis in Preterm Labor. Am J Reprod Immunol.2016;75(2):112-25.
123. Miura Y, Payne MS, Keelan JA et al. Maternal intravenous treatment with either azithromycin or solithromycin clears Ureaplasma parvum from the amniotic fluid in an ovine model of intrauterine infection. Antimicrob Agents Chemother. 2014; 58:5413-20.
124. Acosta EP, Grigsby PL, Larson KB et al. Transplacental transfer of Azithromycin and its use for eradicating intra-amniotic ureaplasma infection in a primate model. J Infect Dis. 2014;209:898-90